Tchola, O. (1963), *Biochem. Z. 338*, 36.
Ingold, C. K., and Wilson, C. L. (1934), *J. Chem. Soc.*, 773.
Johnson, W. C., and Lindsay, A. J. (1939), *Analyst 64*, 440.
Kobes, R. D., Simpson, R. T., Vallee, B. L., and Rutter, W. J. (1969), *Biochemistry 8*, 585.
Meyerhof, O., Lohmann, K., and Schuster, Ph. (1936), *Biochem. Z. 286*, 301, 319.
Ostromisslensky, J. (1910), *Ber. 43*, 197.
Pearson, R. G., and Dillon, R. L. (1950), *J. Am. Chem. Soc. 72*, 3574.
Pearson, R. G., and Dillon, R. L. (1953), *J. Am. Chem. Soc. 75*, 2439.
Richards, O. C., and Rutter, W. J. (1961a), *J. Biol. Chem. 236*, 3177.
Richards, O. C., and Rutter, W. J. (1961b), *J. Biol. Chem. 236*, 3185.
Riordan, J. F., and Christen, P. (1968), *Biochemistry 7*, 1525.
Riordan, J. F., Sokolovsky, M., and Vallee, B. L. (1966), *J. Am. Chem. Soc. 88*, 4104.
Rose, I. A. (1962), *Brookhaven Symp. 15*, 293.
Rose, I. A., and Rieder, S. V. (1955), *J. Am. Chem. Soc. 77*, 5764.
Rose, I. A., and Rieder, S. V. (1958), *J. Biol. Chem. 231*, 315.
Rutter, W. J. (1964), *Federation Proc. 23*, 1248.
Rutter, W. J. (1965), *in* Evolving Genes and Proteins, Bryson, V., and Vogel, H. J., Ed., New York, N. Y., Academic, p 279.
Rutter, W. J., Hunsley, J. R., Groves, W. E., Calder, J., Rajkumar, T. V., and Woodfin, B. M. (1966), *Methods Enzymol. 9*, 479.
Rutter, W. J., and Ling, K. H. (1958), *Biochim. Biophys. Acta 30*, 71.
Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1967) *Biochem. Biophys. Res. Commun. 27*, 20.
Thiers, R. E. (1957), *Methods Biochem. Anal. 5*, 273.
Vallee, B. L., and Wacker, W. E. C. (1969), *Proteins* (in press).
Walters, W. D., and Bonhoeffer, K. F. (1938), *Z. Physik. Chem. A182*, 265.
Warburg, O., and Christian, W. (1943), *Biochem. Z. 314*, 149.
Werner, A. (1909), *Ber. 42*, 4324.
Wheland, G. W., and Farr, J. (1943), *J. Am. Chem. Soc. 65*, 1433.

Purification and Properties of Asparaginase from *Escherichia coli* B*

Helen A. Whelan and John C. Wriston, Jr.

ABSTRACT: *Escherichia coli* B asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) has been purified *ca.* 2000-fold by a combination of heat denaturation, gel filtration, chromatography on DEAE-cellulose, and calcium hydroxylapatite, and polyacrylamide gel electrophoresis. The enzyme appears to be homogeneous, as judged by several criteria, including sedimentation equilibrium ultracentrifugation, disc electrophoresis, and immunoelectrophoresis. At 0.1% protein concentration, the molecular weight by equilibrium sedimentation was found to be *ca.* 139,000. In 8 M urea or 5 M guanidinium hydrochloride, dissociation to an inactive species with an apparent molecular weight of 19,000–24,000 was observed. The amino acid composition has been determined, as well as certain other properties of the enzyme.

In 1961, Broome proposed that the enzyme asparaginase is responsible for the antilymphoma activity of guinea pig serum, an activity first noted by Kidd (1953). This has been confirmed by further work from several laboratories (Mashburn and Wriston, 1963; Broome, 1963; Old *et al.*, 1963; Yellin and Wriston, 1966a,b) and is now generally accepted. Several microorganisms have also been found to contain asparaginase activity (*e.g.*, Varner, 1960) and at least three of these bacterial asparaginases possess antilymphoma activity. An *Escherichia coli* asparaginase was described in 1957 (Tsuji, 1957) and subsequently Mashburn and Wriston (1964) showed that partially purified *E. coli* B asparaginase had antilymphoma activity in mice. The *E. coli* enzyme is currently undergoing extensive clinical trials in human beings (Oettgen *et al.*, 1967; Hill *et al.*, 1967). An asparaginase from *Serratia marcescens* gave results in mice similar to those obtained with the *E. coli* asparaginase (Rowley and Wriston, 1967). Also, Wade *et al.* (1968) recently reported the isolation from *Erwinia carotovora* of an asparaginase with antilymphoma activity in mice. Here we wish to report the isolation from *E. coli* B of a 2000-fold purified asparaginase which is essentially homogeneous by sedimentation equilibrium ultracentrifugation and disc electrophoresis, and to describe certain properties of this enzyme. A preliminary account of this work has appeared (Whelan and Wriston, 1968).

* From the Department of Chemistry, University of Delaware, Newark, Delaware 19711. *Received August 7, 1968.* Supported in part by grants from the National Cancer Institute (CA 06780) and the Damon Runyon Memorial Fund (DRG 660).

Experimental Section

Protein Determination and Asparaginase Assay. Protein concentration was determined routinely according to Waddell (1956) after diluting protein solutions with 0.85% sodium chloride (w/v) or buffer to an optical density at 215 mμ of 1.0 or less (1-cm cell). Measurements were made on the Zeiss PMQ-II spectrophotometer. Occasionally the protein was also determined by the method of Lowry *et al.* (1951) with satisfactory agreement. Standard curves for both methods were prepared with crystalline bovine serum albumin. Protein profiles in column effluents were measured at either 215 or 280 mμ.

Asparaginase activity was determined by direct nesslerization. For routine assay, enzyme solution (1–20 μl) was added to 1.5 ml of 0.05 M Tris (pH 8.5). The reaction was started by adding 0.5 ml of 0.04 M asparagine in the same buffer and allowed to proceed for 30 min at 37°. The reaction was stopped by the addition of 0.1 ml of 1.5 M trichloroacetic acid, centrifuged if necessary, and the mixture was transferred quantitatively to 10-ml graduated test tubes and diluted to 9.5 ml with distilled water. The mixture was then combined with 0.5 ml of Nessler's reagent (Fisher Scientific Co., SO-N-24) and allowed to stand for 15 min at room temperature before estimating ammonia by determining the optical density at 500 mμ with a Spectronic 20 colorimeter. Enzyme and substrate blanks were included in all assays, and a standard curve was prepared with ammonium sulfate. A unit of activity is defined as that amount of enzyme which will catalyze the formation of 1 μmole of ammonia/min under the conditions of the assay (International unit). Specific activity is expressed as units per milligram of protein.

Amino Acid Analysis. Protein was hydrolyzed according to Moore and Stein (1963) in 6 N HCl for 24 and 72 hr at 110 ± 1°. Aliquots containing *ca.* 100 μg of protein were analyzed with the automatic amino acid analyzer of Hamilton (1962, 1963) which consists of a single, high-resolution column capable of determining 0.01 μmole of an amino acid to within ±5%. Values for serine and threonine were extrapolated to zero time, assuming first-order kinetics. Tryptophan was estimated spectrophotometrically according to Beaven and Holiday (1952). Amide nitrogen was not determined.

Total SH groups (half-cystines) were determined with Ellman's (1959) reagent following the procedures of Morino and Snell (1967) by denaturing and reducing disulfide linkages in the protein. About 1 mg of asparaginase was incubated at 37° for 2 hr at pH 10.5 in 1 ml of a solution of 5 M guanidinium chloride, 50 mM EDTA, and 50 mM dithiothreitol buffered with 0.1 M potassium bicarbonate. The mixture was then passed through a Sephadex G-25 column (0.9 × 12 cm) that had been equilibrated at pH 5 with a solution containing 5 M guanidinium chloride, 0.02 M potassium acetate, and 50 mM EDTA. The protein was located in the effluent by absorption at 280 mμ. Titration was started immediately after separation by adding 0.02 ml of 10 mM 5,5′-dithiobis(2-nitrobenzoic)-acid solution in 0.05 M potassium phosphate buffer (pH 7.5). The reaction was followed at 412 mμ in a Cary spectrophotometer, and the number of sulfhydryl groups reacted was based on a molar absorbance of 13,600 for the reduction product of 5-5′-dithiobis(2-bitrobenzoic) acid.

Electrophoresis. Polyacrylamide gel electrophoresis was carried out with the Canalco Model 6 apparatus essentially as described by Davis (1964) and Ornstein (1964). The separating gels (pH 8.9) were 10%, and the sample and stacking gels (pH 6.8) 3.5% acrylamide. Tris-glycine buffer (0.05 M, pH 8.4) was used in both chambers of the apparatus. Electrophoresis was carried out in the coldroom at 4 mA/tube for 45 min. The gels were stained with either Amido-Schwarz or coomassie blue (Chrambach *et al.*, 1967). Electrophoresis in 8 M urea was performed by the method of Reisfeld *et al.* (1962) using gels that were 7.5% acrylamide, stacked at pH 5.0, and run at pH 4.3. Electrophoresis was carried out in the cold at 5 mA/tube for approximately 2 hr. Urea was freshly recrystallized from alcohol.

Preparative-scale polyacrylamide gel electrophoresis was carried out with the Canalco Prep-disc equipment, using their smallest column (PS2/70). The column is prepared by casting a 10% separating gel (6 cm), a stacking gel (3.5%, 2 cm), and a sample gel (3.5%, 5 cm). The column is operated for 10 hr at 4 mA, at 0°, eluting with 0.05 M Tris (pH 8.8).

The isoelectric point was estimated by electrophoresis on cellulose acetate strips in a series of 0.05 M acetate buffers, with pH values ranging from 4.3 to 5.4. Approximately 2 μg of protein was applied to each strip. Electrophoresis was carried out in a Durrum cell at 2 mA/strip for 25 min in the cold room. After drying, the strips were stained with freshly prepared ponceau S (0.5% in 5% trichloroacetic acid) and destained with several washings of 5% acetic acid.

Immunoelectrophoresis was carried out according to the procedures of Campbell *et al.* (1964) on microscope slides in a Durrum cell containing 0.05 M sodium barbital buffer (pH 8.2). The slides were coated with 0.85% Ionagar, and current was passed for 1 hr at 350 V. Protein bands were developed by staining with acid fuchsin.

Sedimentation. Molecular weights were determined by both the Archibald (1947) approach to equilibrium method and the Yphantis (1964) meniscus depletion equilibrium method, using the Spinco Model E ultracentrifuge. Schlieren optics were used to determine concentration gradient and total concentration in the Archibald studies, and Rayleigh interference optics for the Yphantis method. The cells were 12-mm interference cells fitted with either double-sector or double-sector capillary centerpieces. In the Archibald studies, rotor speeds between 6,995 and 42,040 rpm were used, depending upon the protein concentration. In the Yphantis studies, speeds from 14,290 to 33,450 were used. At least two different speeds were used at each protein concentration, and in some cases, in order to diminish the time needed to reach equilibrium, the initial rotor speed selected was approximately one-third in excess of the final speed. As soon as the higher speed was reached, the rotor speed was reduced to the final desired rpm (overshoot method). Photographic plates were analyzed with the aid of a Nikon 6 microcomparator or a Gaertner toolmaker's microscope. Slightly different conditions were used in Yphantis studies carried out in two laboratories, as indicated in the legend for Table II. In all experiments, enzyme was dialyzed at 4° for at least 24 hr *vs.* multiple changes of the appropriate buffer, and buffer from the final change of dialysate was used as reference solution.

Sedimentation velocity was determined from the schlieren patterns of the enzyme photographed at 8- or 16-min intervals after reaching final speed of 42,040 or 50,740 rpm at 20°. Observed sedimentation coefficients were corrected to values corresponding to a solvent with the viscosity and density of

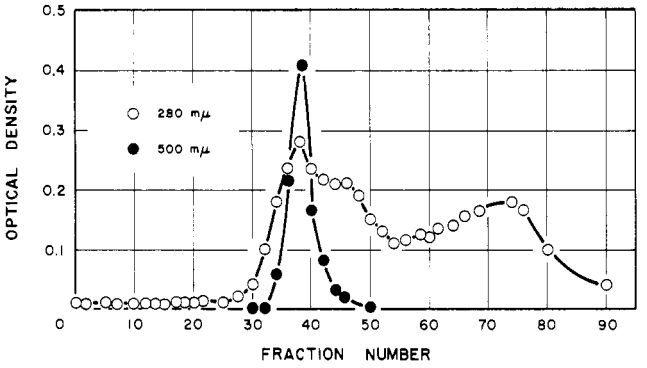

FIGURE 1: Gel Filtration of asparaginase from heat-treated extracts of *E. coli* B. Bio-Gel P-150 was equilibrated with 0.02 M potassium phosphate buffer (pH 8.0) and the same buffer was used for elution at 60 ml/hr. Protein concentration (open circle) is in terms of optical density at 280 mμ; asparaginase activity (closed circles) in terms of optical density at 500 mμ (see text for assay).

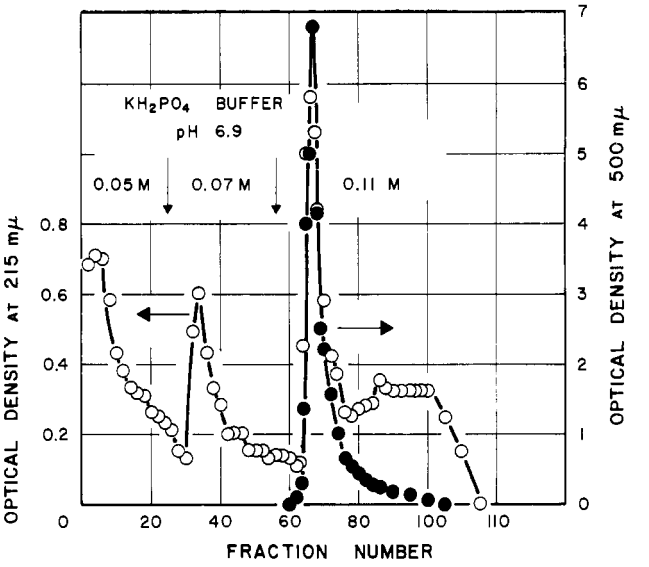

FIGURE 3: Calcium hydroxylapatite chromatography of *E. coli* asparaginase fraction from DEAE-cellulose column. A 2 × 20 cm column was equilibrated with 0.05 M potassium phosphate buffer (pH 6.9). The buffer was changed to 0.07 and 0.11 M potassium phosphate buffers (pH 6.9), respectively, at the points indicated by arrows. Protein concentration (open circles) is in terms of optical density at 215 mμ, and asparaginase activity (filled circles) in terms of optical density at 500 mμ (see text for assay). The column was operated at about 50 ml/hr, and 5-ml fractions were collected.

water at 20° ($s_{20,w}$) and the corrected values were plotted against concentration to obtain $s^0_{20,w}$ values. A 10-ml pycnometer was used for the determination of density of solvents, and the viscosity of the dissociating solvents was determined according to Zimm and Crothers (1962). The partial specific volume of asparaginase was calculated from the amino acid composition (Schachman, 1957), and the value of $\bar{v}$ was reduced by 0.01 cm^3/ml (Tanford *et al.*, 1967) for molecular weight calculation in 8 M urea and 5 M guanidine solvent systems.

Results

Purification of E. coli B Asparaginase. Lyophilized cells (20 g; Worthington Biochemical Co., ECB) are stirred overnight in 100 ml of Tris buffer (0.05 M, pH 8.6). The suspension is then sonicated for 3 min (Branson 20KC sonifer, full power), centrifuged (Servall RC-2, 27,000*g*, 30 min) and the supernatant was set aside. Sonication is repeated twice, resuspending the pellet each time in 40 ml of cold Tris buffer. Nucleic acids are removed from the combined supernatants by adding 1 M $MnCl_2$ (0.05 volume) with stirring over a 10–15-min period and then standing in the cold for 48 hr. After centrifuging as above, the supernatant is heated for 5 min at 55°, immersed in ice, and allowed to stand 1–2 hr. The suspension is again centrifuged as before, and the supernatant is concentrated by ultrafiltration to 20 ml or less. This heat treatment eliminates a second asparaginase, designated EC-1 by Campbell *et al.* (1967), which is present in *E. coli* extracts.

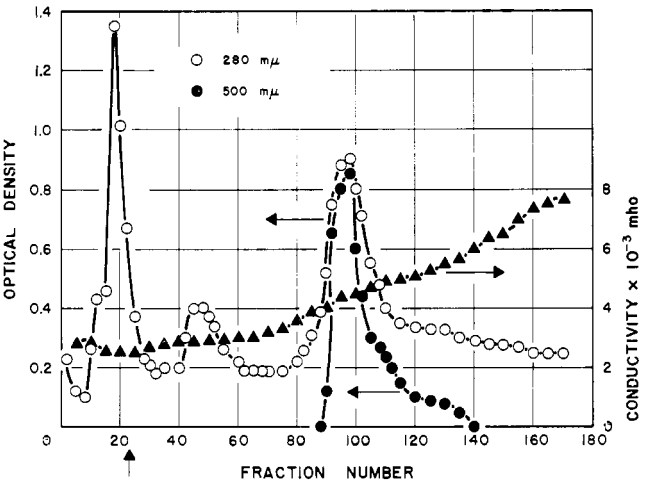

FIGURE 2: DEAE-cellulose chromatography of asparaginase fraction from gel filtration. A 3.0 × 30 cm column was equilibrated with 0.02 M potassium phosphate buffer (pH 8.0). After about 1.5 column volumes of starting buffer, a linear gradient to 0.2 M potassium phosphate buffer (pH 6.35) was started at the point indicated by an arrow. The column was operated at approximately 80 ml/hr, collecting 10-ml fractions. Protein concentration (open circles) in terms of optical density at 280 mμ, asparaginase activity (filled circles) in terms of optical density at 500 mμ (see text for assay), and conductivity is represented by solid triangles.

The protein solution is made 10% in sucrose and layered onto a Bio-Gel P-150 column (5-cm diameter, 1800-2000 ml column volume). The Bio-Gel is equilibrated with 0.02 M potassium phosphate buffer (pH 8.0) and this same buffer is used to elute protein, as shown in Figure 1.

Peak tubes from the Bio-Gel column are pooled and concentrated by ultrafiltration to a volume of about 100 ml. The protein is then applied to a 3.0 × 30 cm DEAE-cellulose column equilibrated with 0.02 M potassium phosphate buffer (pH 8.0). After passing through about 1.5 column volumes of starting buffer, the protein is eluted with a linear gradient from starting buffer to potassium phosphate buffer (0.2 M, pH 6.35) as shown in Figure 2. Peak tubes are again pooled, concentrated by ultrafiltration, and dialyzed against 0.05 M potassium phosphate buffer (pH 6.9) until the conductivity is less than 6.0×10^{-3} mho. The enzyme solution is then applied to a calcium hydroxylapatite column (2.0 × 20 cm) prepared by mixing approximately two volumes of Bio-Rad calcium hydroxylapatite suspension with one part by weight of Whatman cellulose powder. The powder is suspended in starting buffer

TABLE I: Purification of *E. coli* B Asparaginase.[a]

Step	Sample Vol (ml)	Total Protein (mg)	Total Units	Sp Act. (units/mg)	Stepwise Recov (%)
Crude extract	140	12–14	3500	0.2–0.25	
$MnCl_2$, heat	90–100	1800	2400	1.3	65
P-150 Bio-Gel	8–10	1200	2200	1.8	90
DEAE-cellulose	40–50	500–800	1600	20–32	70
Calcium hydroxylapatite	25	3–6	800	150–250	50
Prep-disc electrophoresis	4	1.5–2	600	300–400	90

[a] From 20 g of lyophilized cells. Experimental details are given in the text. Recoveries in ultrafiltration and dialysis were essentially quantitative and these steps are omitted in the table. Over-all purification of approximately 2000-fold, with 15% recovery.

(0.05 M potassium phosphate, pH 6.9) and the hydroxylapatite slurry is added with constant stirring. The column is packed and operated under hydrostatic pressure. The column is developed stepwise, as shown in Figure 3, with asparaginase emerging with the 0.11 M buffer.

Peak tubes are pooled, concentrated by ultrafiltration to 2 ml or less, and dialyzed against 0.005 M potassium phosphate buffer (pH 6.8) before carrying out the preparative-scale polyacrylamide gel electrophoresis. The separation achieved in this step is shown in Figure 4.

Asparaginase-containing fractions from Prep-disc electrophoresis often show a single band on disc electrophoresis, as shown in Figure 5, although some preparations with essentially the same specific activity show a second slower moving faint band. The highest specific activity we have obtained is 400 units/mg, representing a 2000-fold purification with an over-all recovery of *ca.* 15%. Table I presents a summary of the purification scheme.

Molecular weight. The molecular weight was determined on one of our earliest samples by the method of equilibrium sedimentation according to Yphantis (1964) and the logarithm of fringe displacement was plotted against distance from the center of rotation, as shown in Figure 6. The molecular weight was calculated from the slope of the best straight-line fitting the experimental points according to the equation

$$M = \frac{RT}{w^2 r(1 - \bar{v}\rho)} \frac{\mathrm{d} \ln c}{\mathrm{d}r}$$

The value of $\bar{v}$ was calculated from the amino acid composition of the enzyme (Schachman, 1957) and found to be 0.730. The molecular weight determined by this method (apparent weight-average molecular weight) was 139,000, taken as the average of values obtained at 0.1, 0.05, and 0.017% protein concentration (Table II). Evidence for homogeneity is provided by the fact that the plots are linear at two of these concentrations, although there was a slight upward curvature for the sample at 0.1% concentration.

Subunits. Evidence for the existence of subunits has been obtained in further experiments in the ultracentrifuge, reported in detail elsewhere (Kirschbaum *et al.*, 1968). Sedimentation coefficients ($s^0_{20,w}$ values) for asparaginase in several different solvents are shown in Table III.

In the region from 10 to 2 mg per ml, the material was homogeneous (single symmetrical peak) in 0.02 M sodium phosphate buffer–0.2 M NaCl (pH 6.85) with a sedimentation coefficient that increased slightly with dilution. Extrapolation to zero concentration gave an $s^0_{20,w}$ value of 8.6 S, as shown in Table III. At concentrations of 1 mg/ml or below, the material ap-

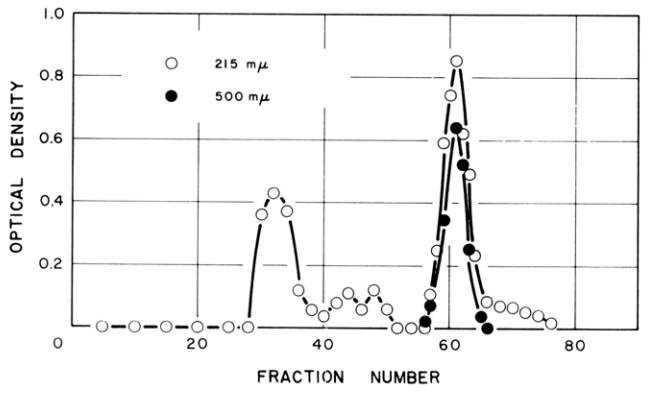

FIGURE 4: Polyacrylamide gel electrophoresis of *E. coli* asparaginase fraction from calcium hydroxylapatite column. (See text for preparation of column.) The column is operated for 10 hr at 4 mA at 0° and eluted with 0.05 M Tris buffer (pH 8.8) collecting 5-ml fractions. Protein concentration (open circles) is in terms of optical density at 215 mμ, and asparaginase activity (filled circles) is in terms of optical density at 500 mμ.

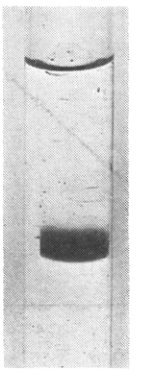

FIGURE 5: Disc electrophoresis of *E. coli* B asparaginase.

TABLE II: Equilibrium Sedimentation Analysis of *E. coli* B Asparaginase.[a]

Sample	Method	Rotor Speed	Mol Wt
Expt 1			
0.1% enzyme in 0.2 M potassium phosphate, pH 7.25, + 1% sucrose	Yphantis	16,200	130,000–144,500
0.05% enzyme in same system	Yphantis	16,200	140,400
0.0167% enzyme in same system	Yphantis	16,200	140,300
Expt 2			
1% enzyme in 0.02 M sodium phosphate–0.2 M NaCl, pH 6.85	Yphantis	14,290; 20,410	255,000 ± 5%
	Archibald	6,995; 8,766; 12,590	
0.1% in same system	Yphantis	14,290; 20,410	125,000 ± 5%
	Archibald	8,766; 12,590	
0.02% enzyme in same system	Yphantis	29,500; 33,450	64,000 ± 3%
	Archibald	8,766; 12,590	
1% enzyme in same system + 8 M urea or 5 M guanidine	Yphantis	29,500; 33,450	19,000–24,000
	Archibald	42,040	

[a] In expt 1, the centrifuge was operated at 16,200 rpm at 20° for 28 hr; in expt 2, at various speeds at 20° as shown. At 0.1% protein concentration in expt 1 there is a slight upward curvature of the plot of log fringe displacement *vs.* protein concentration, and different values for molecular weight are obtained, using the upper or lower portions of the plot to obtain the slope. The average of all four values in expt 1 (apparent weight-average molecular weight) is 138,900.

pears to dissociate, and other species appear (J. Kirschbaum, J. C. Wriston, and O. T. Ratych, in preparation). The single peak with a sedimentation coefficient of about 8.5 S that is characteristic of the enzyme at pH values from 5 to 10 largely disappears at pH 2.2, and a second peak, with a $s^0_{20,w}$ of 4.1 S, appears. If the enzyme is centrifuged in a dissociating solvent such as 8 M urea, a single peak with a sedimentation coefficient of about 1.5 S is seen.

Further evidence for the existence of subunits has been obtained by determining the molecular weight of asparaginase at various concentrations and in dissociating solvents. At 0.1% enzyme concentration, the molecular weight obtained was reasonably close to that determined earlier in a somewhat different buffer, but at 1% asparaginase, a value about twice as high was obtained, and at low protein concentrations (0.02%) the apparent molecular weight is 64,000. The apparent molecular weight of the subunit in 8 M urea or 5 M guanidine is between 19,000 and 24,000. These relationships are also shown in Table II, expt 2. A potential source of error in determining molecular weights in a solvent such as 5 M guanidine is a change in the density of solvent due to redistribution of guanidine. According to Small and Lamm (1966), however, this causes an increase in molecular weight at the bottom of the cell of less than 1%. Some uncertainty also exists concerning the values of partial specific volume in guanidine solutions (Reithel and Sakura, 1963). Values of apparent partial specific volume have been obtained experimentally for γ-globulin (Marler *et al.*, 1964) and myosin (Woods *et al.*, 1963; Kielley and Harrington, 1960) and found to be about 0.01 cc/g below the true values in dilute aqueous salt solutions. Accordingly, the calculated value of partial specific volume of

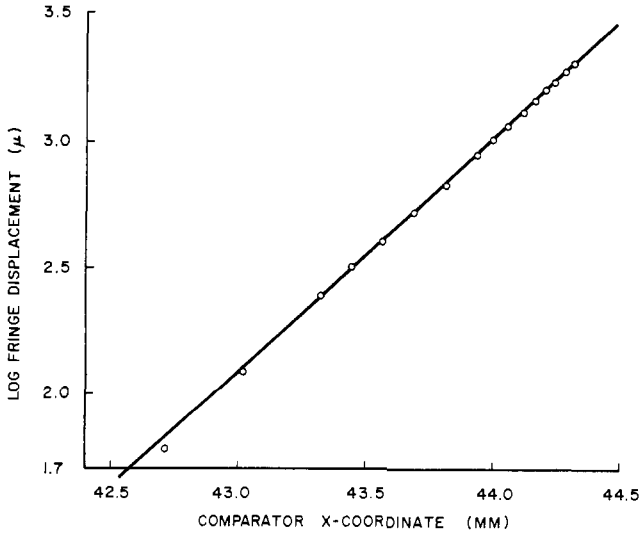

FIGURE 6: Equilibrium sedimentation of *E. coli* asparaginase.

TABLE III: Sedimentation Coefficients of *E. coli* B Asparaginase in Various Solvents.[a]

Sample	$s^0_{20,w}$ (S)
In 0.02 M sodium phosphate–0.2 M NaCl, pH 6.85	8.6
In glycine–HCl buffer, pH 2.2	9S peak (17%) 3.9S peak (83%)
In 0.02 M sodium phosphate–0.2 M NaCl, pH 6.85, and 8 M in urea	1.5

[a] Experimental details are given in the text; protein concentration is 10 mg/ml in all three solvents.

TABLE IV: Amino Acid Composition of *E. coli* B Asparaginase.

Amino Acid	Amino Acid/His		App Min No. of Residues	Residues 133,000 mol wt
	24 hr	72 hr		
Asp	15.1	14.0	15	180
Thr	9.3	8.2	10	120
Ser	4.5	4.4	5	60
Glu	6.8	6.6	7	84
Pro	3.7	3.6	4	48
Gly	9.1	8.9	9	108
Ala	9.7	9.3	10	120
Val	9.4	10.2	10	120
CyS[a]			(0.5)[a]	6
Met	1.9		2	24
Ile	3.6	3.6	4	48
Leu	7.2	7.0	7	84
Tyr	3.3	3.1	4	48
Phe	2.6	2.5	3	36
Lys	6.7	6.1	7	84
His	(1)	(1)	(1)	12
Arg	2.7	2.4	3	36
Trp[b]			1	12

[a] CyS determined by the method of Ellman (see Experimental Section) gave approximately 6 half-cystines/133,000 mol wt. [b] Trp determined by the method of Beaven and Holiday. Minimal molecular weight, 22,170.

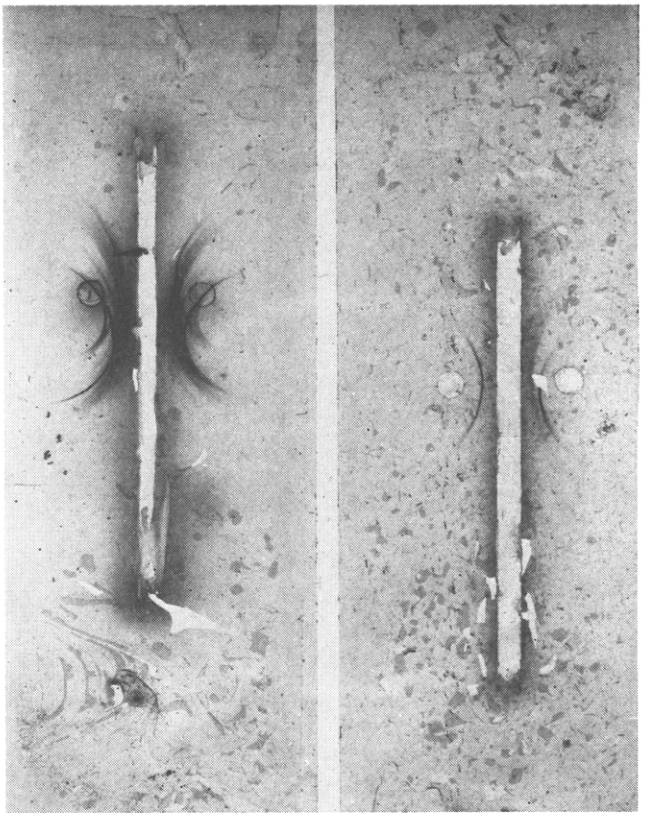

FIGURE 7: Immunoelectrophoresis of *E. coli* B asparaginase. The pattern on the left is produced by crude extract of cells following $MnCl_2$ and heat treatment (see text for experimental details); the pattern on the right is produced by the purified enzyme.

asparaginase obtained from the amino acid composition was reduced by 0.01 (see Tanford *et al.*, 1967, for a discussion).

It has also been possible to demonstrate reversible inactivation of *E. coli* asparaginase with urea. If a dilute enzyme solution (0.05%) is made 6 or 8 M in urea, activity drops immediately to 10% or less of the initial activity (the aliquots withdrawn for asparaginase assay are of such a size that the concentration of urea in the assay system is below 0.1 M). If the urea is removed by dialysis, however, activity is completely restored within 8 hr.

Urea also causes a marked change in disc electrophoresis patterns. At both pH 4.3 and 8.9, the single asparaginase band is replaced by two bands, both of which migrate more slowly than the asparaginase band does in the absence of urea. In the case of disc electrophoresis at pH 8.9 in the presence of urea, the two bands dissociate further if electrophoresis is carried on for a longer time (2 hr instead of 1.25 hr) and four bands can be seen. None of these bands show enzyme activity when unstained comparison gels are sliced and protein eluted.

Amino Acid Composition. The amino acid composition has been determined (see Methods). The amino acids were normalized to histidine, and the values in the fourth column of Table IV represent the apparent minimum number of residues, assuming one histidine. Difficulty was encountered in obtaining reproducible values for cystine on the amino acid analyzer, or cysteic acid following performic acid oxidation according to the method of Hirs (1967) due in part, at least, to the low levels of this amino acid in the enzyme and the limited amounts of material available. Accordingly, the spectrophotometric method of Ellman (1959) was used to estimate cystine. The data obtained in two separate experiments indicate that there is only half of a half-cystine per histidine, and hence the minimal molecular weight of 22,170 was obtained by doubling the values in column 4. The value of 22,170 multiplied by 6, gives a molecular weight for the enzyme of 133,000, a value which is consistent with the molecular weight obtained by ultracentrifugation at 0.1% protein concentration.

Discussion

Evidence for an association between the antilymphoma and asparaginase activities of guinea pig serum was first presented by Broome (1961). Confirmation of this came from several laboratories (Mashburn and Wriston, 1963; Broome, 1963; Old *et al.*, 1963) and in 1964 it was shown by Yellin and Wriston that guinea pig serum asparaginase that was essentially homogeneous had antilymphoma activity. The sera of certain animals related to the guinea pig have since been found to contain asparaginase (Old *et al.*, 1963) and the agouti in particular has been a convenient source of enzymes for experimental purposes. One patient has been treated with guinea pig serum asparaginase (Dolowy *et al.*, 1966) but the low level of this enzyme in guinea pig serum, and the relative unavailability of guinea pig or agouti serum made it impractical to carry out toxicity studies in larger animals and clinical trials in man. These became feasible with the discovery that an asparaginase from *E. coli* B also has antilymphoma activity (Mashburn and

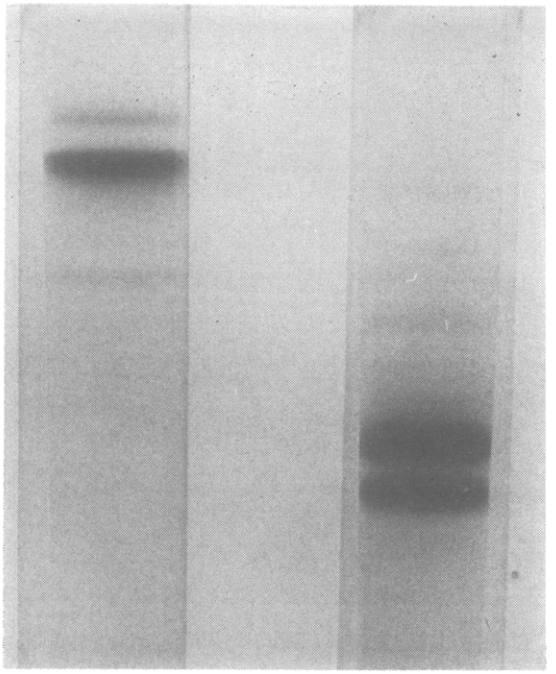

FIGURE 8: Effect of ethanol on disc electrophoretic pattern of *E. coli* asparaginase. The pattern on the right shows the effect of ethanol precipitation (see text for experimental details).

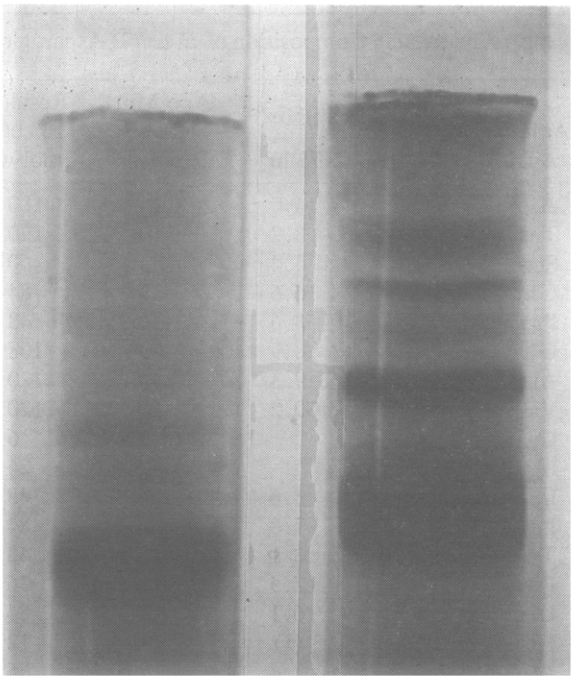

FIGURE 9: Effect of lyophilization on disc electrophoretic pattern of *E. coli* asparaginase. The pattern on the right shows the effect of lyophilization (see text for experimental details).

Wriston, 1964). The purification procedure described here has led to a preparation which appears to be homogeneous on the basis of several criteria: (1) equilibrium sedimentation by the method of Yphantis (1964); (2) the migration of a single symmetrical peak in sedimentation velocity experiments; (3) the formation of a single band in polyacrylamide gel electrophoresis; and (4) the appearance of a single arc upon immunoelectrophoresis (Figure 7). The amino acid analyses are also compatible with a homogeneous protein with a molecular weight of *ca.* 139,000.

There are two asparaginases in *E. coli* B cells, designated EC-1 and EC-2 by Campbell *et al.* (1967). Only EC-2 has antilymphoma activity. The enzymes differ in several other respects, including a greater heat sensitivity on the part of EC-1, and this has been taken advantage of to remove EC-1 in the work described here. The enzymes may also be separated by DEAE chromatography (Campbell *et al.*, 1967). In addition to possessing antilymphoma activity, *E. coli* asparaginase also interferes with the synthesis of protein in microbial extracts under the direction of phage f2 *in vitro* (Schwartz, 1965), inhibits the early mitosis of regenerating livers in rats (Becker and Broome, 1967), and exhibits an embryotoxic effect in rabbits (Adamson and Fabro, 1968).

The enzyme has a broad pH–activity profile, with a maximum at about pH 8. The isoelectric point has been found to be approximately 4.85. Routine asparaginase assays are made at 1×10^{-2} M L-asparagine, but the enzyme is still saturated at 1×10^{-4} M substrate. An apparent K_m of 1.25×10^{-5} has been reported by Broome (1968). Detailed kinetic studies are now in progress in our laboratory.

The molecular weight of the enzyme at 0.1% protein concentration is *ca.* 139,000, but at 1% protein concentration the apparent molecular weight is 255,000, and in dilute solution (0.01%) or in 5 M NaCl, dissociation to a species with an apparent molecular weight of *ca.* 64,000 occurs. Exposure of the enzyme to dissociating solvents such as 8 M urea or 5 M guanidine leads to the appearance of material with an apparent molecular weight of 19,000–24,000, suggesting that the species with an apparent molecular weight of 255,000 is composed of 12 subunits. These subunits do not have enzymatic activity.

An essentially homogeneous preparation of asparaginase from *E. coli* has been reported recently by Roberts *et al.* (1968a,b). This enzyme appears to differ somewhat from our preparation, although it has not yet been possible to make a detailed comparison. The principal differences that have been noted have to do with the isoelectric point and with the specific activity of highly purified material. The isoelectric point of the *E. coli* B enzyme described here is 4.85, determined as described in the Experimental Section by paper strip electrophoresis. Roberts *et al.* (1968b) report an isoelectric point of 4.35, however. With respect to maximum specific activity, our best preparation, giving a single band on disc electrophoresis, had a specific activity of approximately 400 IU/mg, whereas Roberts reported a maximum specific activity of 620 IU/mg.

These differences may be accounted for in several ways. In the first place, different *E. coli* strains were used in the two laboratories. We used an *E. coli* B strain, carried in the collection of the Department of Biological Sciences here and originally obtained from Dr. W. F. Goebel of the Rockefeller Institute; Roberts used an *E. coli* "HAP" strain, isolated in his laboratory. It is thus possible that the two asparaginases are inherently different.

The differences between the two preparations could also be due to differences in the purification procedures. In the Roberts procedure, ethanol is used at two different stages, whereas it is not used at all in the procedure described here. In Roberts' procedure, there is an alcohol fractionation step following rupture of the bacterial cells; and later, after DEAE chromatog-

raphy, the enzyme is precipitated at pH 5.0 by the addition of 1.2 volumes of ethanol. Ethanol may modify the properties of asparaginase, either directly, by partial denaturation, or indirectly by the removal of a bound component, possibly a lipid. The following observations appear to support this suggestion. A sample of *E. coli* asparaginase, taken just after the DEAE step in our procedure, and having a specific activity of 70 IU/mg, was subjected to alcohol fractionation according to Roberts. The enzyme was dissolved in 0.05 M acetate buffer (pH 5.0) and 0.5 volume of ethanol was added in the cold; 0.75 volume of ethanol was then added to the supernatant after centrifugation, and the precipitate obtained was redissolved in 0.02 M potassium phosphate buffer (pH 8.0). The specific activity of the asparaginase increased about twofold (to 135 IU/mg) with 80% recovery, but the properties of the enzyme were changed in two ways.

The material now had a markedly different mobility in disc electrophoresis, moving faster than the untreated enzyme at pH 8.9, as may be seen in Figure 8. In addition, its stability was sharply reduced, in that the alcohol-treated *E. coli* B asparaginase lost most of its activity upon storage in the cold for a week, whereas the same enzyme, prepared without exposure to ethanol, whether of high or low specific activity, may be stored in the cold for long periods of time without sgnificant loss of activity.

Evidence has been presented here for the existence of subunits in *E. coli* asparaginase, but questions concerning their number and arrangement, and whether or not they are identical, remain the subject of future research. The number of bands formed on disc electrophoresis in urea seems to depend strongly upon the conditions used. To complicate the picture further, lyophilization also leads to a marked change in electrophoretic mobility, as shown in Figure 9. Several of the bands that appear following lyophilization of highly purified enzyme have asparaginase activity, as shown by gel slicing and elution experiments.

Acknowledgment

We are greatly indebted to L. L. Anderson of the Dupont Co. and J. Kirschbaum of the Squibb Institute for Medical Research for help with the ultracentrifugal analyses, and to P. B. Hamilton, Alfred I. Dupont Institute, Wilmington, Del., for assistance with the amino acid analyses.

References

Adamson, R. H., and Fabro, S. (1968), *Nature 218*, 1164.
Archibald, W. J. (1947), *J. Phys. Colloid Chem. 51*, 1204.
Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 375.
Becker, F. F., and Broome, J. D. (1967), *Science 156*, 1602.
Broome, J. D. (1961), *Nature 191*, 1114.
Broome, J. D. (1963), *J. Exptl. Med. 118*, 99, 121.
Broome, J. D. (1968), *Trans. N. Y. Acad. Sci. 30*, 690.
Campbell, D. H., Garvey, J. S., Cremer, N. G., and Sussdorf, D. H. (1964), Methods in Immunology, New York, N. Y., Benjamin, p 143.
Campbell, H. A., Mashburn, L. T., Boyse, E. A., and Old, L. J. (1967), *Biochemistry 6*, 721.
Chrambach, A., Reisfeld, R. A., Wyckoff, M., and Zaccharri, J. (1967), *Anal. Biochem. 20*, 150.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Dolowy, W. C., Cornet, J., Henson, B., and Ammeraal, K. (1966), *Proc. Soc. Exptl. Biol. Med. 123, 133.*
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
Hamilton, P. B. (1962), *Ann. N. Y. Acad. Sci. 102*, 55.
Hamilton, P. B. (1963), *Anal. Chem. 35*, 2055.
Hill, J. M., Roberts, J., Loeb, E., Khan, A., MacLellan, A., and Hill, R. W. (1967), *J. Amer. Med. Assoc. 202*, 116.
Hirs, C. H. W. (1967), *Methods Enzymol. 11*, 59.
Kidd, J. G. (1953), *J. Exptl. Med. 98*, 565, 582.
Kielley, W. W., and Harrington, W. F. (1960), *Biochim. Biophys. Acta 41*, 401.
Kirschbaum, J., Wriston, J. C., Jr., and Ratych, O. T. (1968), 156th National Meeting of the American Chemical Society, Sept, Atlantic City, N. J.
Lowry, O. H., Rosebrough, N. J., Farr, A. J., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Marler, E., Nelson, C. A., and Tanford, C. (1964), *Biochemistry 3*, 279.
Mashburn, L. T., and Wriston, J. C., Jr. (1963), *Biochem. Biophys. Res. Commun. 12*, 50.
Mashburn, L. T., and Wriston, J. C., Jr. (1964), *Arch. Biochem. Biophys. 105*, 451.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.
Morino, Y., and Snell, E. E. (1967), *J. Biol. Chem. 242*, 5602.
Oettgen, H. F., Old, L. J., Boyse, E. A., Campbell, H. A., Philips, F. S., Clarkson, B. D., Tallal, L., Leeper, R. D., Schwartz, M. K., and Kim, J. H. (1967), *Cancer Res. 27*, 2619.
Old, L. J., Boyse, E. A., Campbell, H. A., and Daria, G. M. (1963), *Nature 198*, 801.
Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature 195*, 28.
Reithel, F. J., and Sakura, J. D. (1963), *J. Phys. Chem. 67*, 2497.
Roberts, J., Burson, G., and Hill, J. M. (1968a), *J. Bacteriol. 95*, 2117.
Roberts, J., Liu, Y. P., Burson, G., Hill, J. M., and Speer, R. J. (1968b), 156th National Meeting of the American Chemical Society, Sept, Atlantic City, N. J.
Rowley, B., and Wriston, J. C., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 160.
Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.
Schwartz, J. H. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 1133.
Small, P. A., and Lamm, M. E. (1966), *Biochemistry 5*, 259.
Tanford, C., Kawahara, K., and Lapanje, S. (1967), *J. Amer. Chem. Soc. 89*, 729.
Tsuji, Y. (1957), *Naika Hokan 4*, 222; *Chem. Abst. 53*, 10350.
Varner, J. E. (1960), *Enzymes 4*, 243.
Waddell, W. J. (1956), *J. Lab. Clin. Med. 48* 311.
Wade, H. E., Elsworth, R., Herbert, D., Keppie, J., and Sargeant, K. (1968), *Lancet*, 776.
Whelan, H., and Wriston, J. C., Jr. (1968), *Fed. Proc. 27*, 586.
Woods, E. F., Himmelfarb, S., and Harrington, W. F. (1963), *J. Biol. Chem. 238*, 2374.
Yellin, T. O., and Wriston, J. C., Jr. (1966a), *Biochemistry 5*, 1605.
Yellin, T. O., and Wriston, J. C., Jr. (1966b), *Science 151, 998.*
Yphantis, D. A. (1964), *Biochemistry 3*, 297.
Zimm, B. H., and Crothers, D. N. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 905.