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Threonine-Sensitive Aspartokinase–Homoserine Dehydrogenase Complex, Amino Acid Composition, Molecular Weight, and Subunit Composition of the Complex†

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ABSTRACT: The threonine-sensitive aspartokinase–homoserine dehydrogenase complex has been purified to apparent homogeneity from *Escherichia coli* 9723 (ATCC). The amino acid composition of the complex was determined and was found to be similar to that of a complex having similar activities previously isolated from *E. coli* K-12 except in the content of threonine. The molecular weight of the complex determined by sedimentation equilibrium and by the Svedberg method was 346,000 ± 15,000 and 334,000 ± 14,000, respectively. Sedimentation equilibrium experiments performed on the complex dissolved in 6 M guanidinium chloride showed that the molecular weight of the subunit was 80,000 ± 3000. Nonideal behavior of the solute must be taken into account when evaluating the experiments. It was concluded that the complex from *E. coli* 9723 is composed of four subunits of similar or identical molecular weight.

A complex enzyme having both aspartokinase and homoserine dehydrogenase activity and subject to inhibition by the eventual end product, L-threonine, occurs in *Escherichia coli*. The physical and catalytic properties of the complex from strain K-12 have been studied extensively and have been recently reviewed (Cohen, 1969). The native complex isolated from this strain was reported to have a molecular weight of 360,000 (Truffa-Bachi *et al.*, 1968). The molecular weight of the subunits of the complex in 6 M guanidinium chloride was reported to be 60,000, and it was concluded that the complex was composed of six subunits of identical molecular weight (Truffa-Bachi *et al.*, 1969). Recently, subcomplexes having an intermediate molecular weight of 122,000 have been reported (Wampler *et al.*, 1970). These subcomplexes, which seem to occur in certain buffer systems, appear to remain fully effective for both activities of the native complex, and both activities remain sensitive to threonine inhibition.

The threonine-sensitive aspartokinase–homoserine dehydrogenase complex from *E. coli* 9723 (ATCC) has been studied in this laboratory, particularly with respect to the control of biosynthesis of the complex and the threonine interactions with the complex (Lee *et al.*, 1966; Cunningham *et al.*, 1968). Preliminary work on the molecular weight of the complex and subunits indicated that the reported molecular weight of the complex and subunits from E. *coli* K-12 might not be adequate to explain the properties of the complex from strain 9723. Therefore, more complete studies of the physical properties of the complex from strain 9723 have been performed.

In the present work, the complex from *E. coli* 9723 has been purified to homogeneity, and the amino acid composition, the molecular weight, some general physical properties of the complex, and the molecular weight of the subunits of

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the complex have been determined. Careful consideration has been given in this study to the nonideality of solutions of proteins in concentrated guanidinium chloride (Munk and Cox, 1972).

Materials and Methods

Materials

All amino acids (L isomers unless otherwise specified), 2-mercaptoethanol, dithiothreitol, streptomycin sulfate, and Tris buffer were obtained from both Sigma Chemical Co. and Calbiochem, Inc. P-L Biochemicals, Inc., was the source of $NADP^+$, NADPH, and ATP. Sephadex G-200 and DEAE-Sephadex were purchased from Pharmacia Fine Chemicals, and modified hydroxylapatite (Hypatite-C) was obtained from Clarkson Chemical Co. Acrylamide, *N,N'*-methylenebisacrylamide, and riboflavin were purchased from Eastman Chemicals, Canalco Industries, and Merck and Co., Inc., respectively. Matheson Coleman & Bell was the source of *N,N,N',N'*-tetramethylethylenediamine, ammonium persulfate, bromophenol blue, and aniline black. Phenazine methosulfate, *p*-nitrotetrazolium blue, Enzyme grade ammonium sulfate, and Ultra Pure grade guanidinium chloride were purchased from Schwarz-Mann. Protein standards for gel electrophoresis were obtained from Worthington, Sigma Chemical Co., and P-L Biochemicals, Inc. Sodium dodecyl sulfate was purchased from Nutritional Biochemicals, Inc. All inorganic chemicals not previously mentioned were J. T. Baker reagent grade. Aspartic β-semialdehyde was prepared from DL-allylglycine by ozonolysis (Black and Wright, 1955a). The solution was stored in 4 N HCl at −20° and was neutralized with 50% KOH before use. Deionized water was used for all solutions.

Assays

Aspartokinase Assay. Aspartokinase activity was assayed by measuring the absorption of visible light (540 nm) of the asparto-β-hydroxamate–iron complex resulting from the interaction of the product, β-aspartyl phosphate, with hydroxylamine and ferric chloride (Black and Wright, 1955b; Stadtman *et al.*, 1961; Lee *et al.*, 1966). In a total volume of 1 ml, the components of the reaction mixture (pH 8.0) were ATP (10 mM), magnesium chloride (1.6 mM), Tris (100 mM), hydroxylamine hydrochloride (800 mM), aspartic acid (10 mM; absent in control tubes), and rate-limiting enzyme. Tubes containing the mixture were kept in ice until the addition of enzyme, and the reaction was initiated by placing the tubes in a water bath at 30°. After 20-min incubation, the reaction was stopped by adding to each tube 3 ml of acidic ferric chloride reagent (Hoagland *et al.*, 1956) of the following composition: 100 g of $FeCl_3 \cdot 6H_2O$, 50 g of trichloroacetic acid, 46.7 ml of 12 N HCl, and enough water to make 1 l. of solution. The optical density of the solution was measured, and a standard curve was used to obtain the activity of the aspartokinase in micromoles of β-aspartyl phosphate per minute. The units of specific activity of the aspartokinase are micromoles of β-aspartyl phosphate per minute per milligram of protein.

Homoserine Dehydrogenase Assays. The homoserine dehydrogenase activity in the forward (hydrogenation of aspartic β-semialdehyde to produce homoserine) direction was determined by observing the decrease in optical density (340 nm) resulting from the oxidation of NADPH (Patte *et al.*, 1966; Black and Wright, 1955c). In a total volume of 1 ml, the components of the reaction mixture (pH 7.2) were NADPH (0.1 mM), aspartic β-semialdehyde (0.8 mM), Tris (100 mM), potassium chloride (80 mM), and rate-limiting enzyme. Control reactions were carried out in the absence of aspartic β-semialdehyde. The temperature of the assay was 27 ± 0.5°. The rate of the reaction was linear over the first minute, and the change in optical density per minute was converted with the molar extinction coefficient of NADPH, 6.2×10^3 (Kornberg and Horecker, 1953), to concentration of NADPH oxidized. The units of specific activity of the enzyme in the forward direction are micromoles of NADPH oxidized per minute per milligram of protein.

The homoserine dehydrogenase activity in the reverse direction (dehydrogenation of homoserine to produce aspartic β-semialdehyde) was determined by observing the increase in optical density (340 nm) resulting from the reduction of $NADP^+$ (Cunningham *et al.*, 1968). In a total volume of 1 ml, the components of the reaction mixture (pH 8.8) were $NADP^+$ (0.4 mM), homoserine (50 mM), Tris (100 mM), potassium chloride (80 mM), and rate-limiting enzyme. Controls contained no homoserine. The temperature was 27 ± 0.5°. The rate was linear over the first minute, and the amount of NADPH produced per minute was calculated from the extinction coefficient of NADPH. The units of specific activity of the homoserine dehydrogenase in the reverse direction are micromoles of NADPH produced per minute per milligram of protein.

Protein Assays. Protein in fraction I was assayed according to Lowry (Lowry *et al.*, 1951), while all other fractions were assayed with the optical density ratio method (Warburg and Christian, 1942).

Purification Procedures

Buffers. A solution of potassium phosphate (0.02 M) and 2-mercaptoethanol (0.03 M) was the primary buffer used in the preparation of the enzyme complex. In addition to these two components, potassium chloride was present in buffers A, C, and D (0.10 M), in buffer E (0.22 M), and in buffer F (0.33 M); buffer B contained no potassium chloride. Threonine was added to buffers A, B, E, and F (0.002 M), to buffer C (0.005 M), and to buffer D (0.02 M). Buffers A, B, C, and D were at pH 6.8, while buffers E and F were at pH 7.2. Buffer G was potassium phosphate (0.10 M, pH 7.0), guanidinium chloride (6 M), and dithiothreitol (0.05 M).

Preparation of Bacteria. Cells of *E. coli* 9723 were grown at 37° in aerated batch culture in Anderson's salts–glucose medium (Anderson, 1946). The basal medium was supplemented with 0.5 mM lysine and 0.5 mM methionine. Growth was monitored by the increase in optical density (660 nm) of the suspension of bacteria, and when the optical density reached 0.50–0.60, *i.e.*, about 0.314–0.376 mg dry weight of cells per ml, the cells were harvested by centrifugation in a Sharples continuous-flow centrifuge. The harvested cells were washed twice in buffer A by resuspension and centrifugation, and immediately after washing the cells were frozen at −20° until used. A typical preparation involved the harvest of 80–100 g dry weight of cells.

Purification of the Enzyme. A summary of the purification is shown in Table I. At several indicated stages during the purification, the protein was concentrated by precipitation from 50% saturated (7°) ammonium sulfate and recovered by centrifugation at 15,000–30,000*g* (0°). Other centrifugation steps were at the same conditions. The column procedures were performed at 7°, and the buffers were stored and used at 7°.

Fraction I was the bacterial cell homogenate prepared from

TABLE I: Summary of the Purification of the Threonine-Sensitive Aspartokinase–Homoserine Dehydrogenase Complex.[a]

Fraction	Total Protein (mg)	Total Units of Aspartokinase (μmoles/min)	Sp Act. (units/mg)	% Yield	Purification	Total Units of Hse Dehydrogenase (μmoles/min)	Sp Act. (units/mg)	% Yield	Purification
I. Homogenate	65,000	4600	0.0701	100.0	1.00	2310	0.0352	100.0	1.00
II. 0–50% $(NH_4)_2SO_4$	20,700	2510	0.121	54.6	1.73	1290	0.0623	55.8	1.77
III. Heat treatment	9,010	1820	0.202	39.6	2.88	939	0.104	40.6	2.95
IV. G-200 Sephadex	6,630	1830	0.276	39.8	3.94	900	0.136	39.0	3.86
V. DEAE-Sephadex	886	2090	2.36	45.4	33.7	1030	1.16	44.6	33.0
VI. Hydroxylapatite	93.4	1080	11.6	23.5	165	531	5.69[b]	23.0	162

[a] The homoserine dehydrogenase activity reported is for the dehydrogenation direction. The average ratio of the specific activity of aspartokinase to the specific activity of the homoserine dehydrogenase is 2.0 with the experimental deviation ±0.06. [b] The specific activity of fraction VI in the hydrogenation reaction was 44.4 units/mg. When the hydrogenation reaction is studied in phosphate buffer rather than in Tris buffer as it was here, the specific activity was increased by a factor greater than two.

87 g dry weight of frozen cells which were thawed, resuspended in about 1.5 l. of buffer A, and ruptured at 8000 psi in a precooled Gaulin laboratory homogenizer.

Cell debris was removed from fraction I by centrifugation and was discarded. The supernatant was treated with streptomycin sulfate (final concentration, 1%) to precipitate the nucleic acids; the nucleic acids were removed by centrifugation; and the protein was recovered from the supernatant as indicated above. The pellet of protein was resuspended in and was dialyzed against buffer C to obtain fraction II.

Fraction II was heated at 50° for 10 min, and the precipitated protein was removed by centrifugation and discarded. Protein was recovered from the supernatant; the pellet was resuspended in buffer A and was dialyzed against buffer A to obtain fraction III.

Fraction III was further purified by chromatography on G-200–120 Sephadex (column dimensions, 5.0 × 88 cm; void volume, V_0, 565 ml with Blue Dextran; buffer A; upward flow, 0.53 ml/min; 20-ml fractions). Protein in the eluate was detected by the absorption of ultraviolet light (280 nm), and fractions containing protein were assayed for the enzymatic activities. The average $V_e:V_0$ ratio for the elution of the active fractions was 1.19. The active fractions were pooled, and the protein was recovered from them as indicated above. The pellet was resuspended in and was dialyzed against buffer E to obtain fraction IV.

Fraction IV was further purified by ion-exchange chromatography on a column of A-50-120 DEAE-Sephadex (column dimensions, 5.0 × 80 cm; flow rate, 0.44 ml/min; 20-ml fractions). The column was eluted with a linear salt gradient from buffer E (1500 ml) to buffer F (1500 ml). Protein in the fractions was detected by the absorption of ultraviolet light (280 nm), and the fractions containing protein were assayed for enzymatic activity. The activities were eluted from the column between 0.24 and 0.29 M potassium chloride. The active fractions were pooled; the protein was recovered from them as indicated above; and the pellet was resuspended in and was dialyzed against buffer B to obtain fraction V.

Fraction V was placed on a column of hydroxylapatite (column dimensions, 3.5 × 30 cm) which had been equilibrated with buffer B. The column was then eluted with 1 l. of buffer B, and the protein was recovered from the eluate as before. The pellet was resuspended in and dialyzed against buffer D to obtain fraction VI, an aliquot of which was assayed. Fraction VI contains a very small percentage of a protein which sediments more slowly than the native enzyme complex. This protein was removed by chromatography through a small column of G-200 Sephadex before any physical or chemical measurements were made. No significant purification was observed in this step. The complex thus prepared is homogeneous in disc gel electrophoresis experiments (Figure 1) and in sedimentation velocity experiments (Figure 2), and may be stored in buffer D or as an ammonium sulfate precipitate for a period of time. However, aggregation of the purified complex has been observed during storage by both methods. Removal of the aggregates may be achieved by chromatography on G-200 Sephadex prior to any physical study on stored enzyme.

Amino Acid Analysis

Preparation of the Complex. A solution containing about 12 mg of the complex was dialyzed extensively against water, and the resulting suspension of protein was dried under

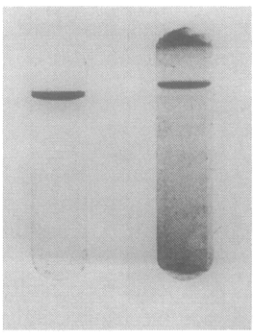

FIGURE 1: Polyacrylamide gel electrophoresis of the purified complex (about 75 μg/gel) in the presence of threonine. Gel A was stained for protein and gel B was stained for homoserine dehydrogenase activity.

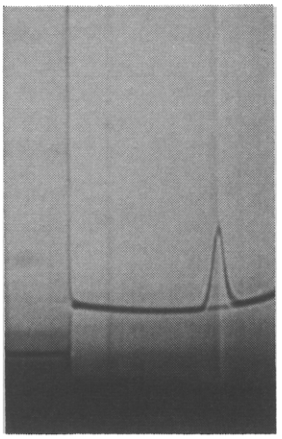

FIGURE 2: A sedimenting boundary of the purified complex during sedimentation velocity experiments at 60,000 rpm. The protein concentration was 6.0 mg/ml.

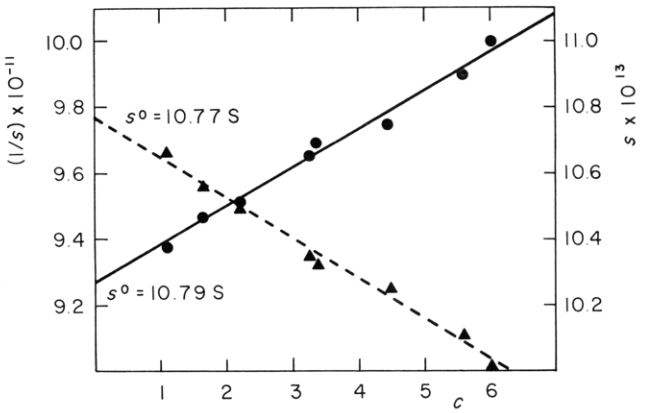

FIGURE 3: The dependence of the sedimentation coefficient of the complex upon concentration. Both the extrapolation of s (▲) and $1/s$ (●) were linear, and the intercept was identical within experimental error for both plots. Units of s are reciprocal seconds, and units of c are mg/ml.

vacuum. The residue was divided into two equal parts for oxidation and for direct hydrolysis of the native complex.

Oxidation of the Complex. The residue obtained as outlined was oxidized essentially according to the procedure of Hirs (1967).

Acid Hydrolysis of the Complex. The procedure for acid hydrolysis of oxidized complex was essentially that of Stein and Moore (1963). A procedure which has been reported to permit recovery of about 90% of the tryptophan content of proteins (Matsubara and Sasaki, 1969) was used for hydrolysis of the native complex. Oxidized protein was hydrolyzed in 6 N HCl, while the native complex was hydrolyzed in 6 N HCl containing 4% mercaptoacetic acid. In each case, at least four samples of the protein were prepared in sealed, evacuated tubes, and hydrolysis of the protein proceeded for 18, 24, 36, or 48 hr at 110°.

Amino Acid Analysis. Two instruments based on the automatic techniques of Spackman, Stein, and Moore, a Beckman–Spinco Model MS analyzer and a Phoenix Model M-7800 analyzer, were used to obtain analyses of the amino acid content of the complex (Spackman *et al.*, 1958; Moore *et al.*, 1958). Calibration constants for the instrument were obtained from a series of analyses using a standard mixture of amino acids. β-2-Thienylalanine and L-α-amino-β-guanidinopropionic acid were used as internal standards on each instrument.

Calculation of the Partial Specific Volume, $\bar{v}$. The partial specific volume of the protein, 0.735 cm³/g, was estimated from the amino acid composition of the protein (Cohn and Edsall, 1943; McMeekin and Marshall, 1952). The effective specific volume, $\bar{v}$*, in 6 M guanidinium chloride was assumed to be 0.725 cm³/g. (See Reisler and Eisenberg, 1969, for the considerations which justify this reduction.)

Disc Gel Electrophoresis

Analytical Disc Gel Electrophoresis. Polyacrylamide gels (0.8 × *ca.* 5.0 cm) were prepared essentially according to the procedure of Davis (1964). Separating and stacking gels were 7.0% polyacrylamide with 0.18% cross-linking (pH 8.9, Tris) and 2.5% polyacrylamide with 0.63% cross-linking (pH 6.7, Tris), respectively. Ammonium persulfate and riboflavin were the respective polymerizing catalysts. The electrode buffer was Tris-glycine (pH 9.5). Both the gels and the electrode buffer contained threonine (2 mM). Bromophenol blue (0.005%) was the tracking dye, and the current during electrophoresis was 5 mA/gel. Protein bands were detected by staining the gel in a 0.5% solution of aniline black in 7% acetic acid for 1–4 hr followed by electrolytic removal of excess dye. Homoserine dehydrogenase activity was detected by incubating the gels in the dark in a solution containing 0.1 mmole/ml of Tris (pH 8.8), 0.05 mmole/ml of homoserine, 0.4 μmole/ml of $NADP^+$, 37 μg/ml of phenazine methosulfate, and 0.59 mg/ml of nitrotetrazolium blue. When distinct purple bands appeared, the reaction was terminated by soaking the gels in 7% acetic acid. The gels stained for homoserine dehydrogenase activity and for protein were stored in 7% acetic acid until photographed.

Sodium Dodecyl Sulfate Gel Electrophoresis. Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate was done as indicated by Weber and Osborn (1969), except that the dialysis step was omitted. The molecular weights of the standard proteins were taken from Klotz and Darnall (1969). About 50 μg of protein was placed on each gel.

Hydrodynamic Measurements

The sedimentation coefficient and the diffusion coefficient of the complex were measured using a Beckman–Spinco Model E analytical ultracentrifuge (schlieren optics). Double-sector cells equipped with sapphire windows were used for most experiments. However, some velocity experiments at high speed were performed using cells equipped with quartz windows. The photographic plates were measured with a Gaertner microcomparator.

Sedimentation Coefficient. In several experiments involving different concentrations of the complex in buffer D, apparent values of the sedimentation velocity, s, were measured according to the procedures indicated by Schachman (1957). The apparent values of s, or alternatively $1/s$, were plotted *vs.* concentration and extrapolated linearly to zero concentration (Figure 3). Both plotting techniques provided virtually the same intercept, s^0, and the standard sedimentation coefficient, $s^0_{20,w}$, was calculated from the average value of s^0 and the viscosity and density of the buffer. The average value obtained from two such determinations of $s^0_{20,w}$ was 11.4 ± 0.1 S.

Diffusion Coefficient. The apparent diffusion coefficient of the native complex was measured at several different concentrations of the complex in buffer D. Schlieren photographs of both sedimentation velocity experiments and synthetic

boundary experiments were utilized and evaluated according to Schachman (1957). Values of D_{app} from both types of experiments were extrapolated linearly to zero concentration (Figure 4), and the average D^0 from both extrapolations was corrected using the viscosity of the buffer relative to water to obtain the standard diffusion coefficient, $D^0_{20,w}$. The average value obtained from these experiments was 3.13×10^{-7} $cm^2\ sec^{-1}$.

Sedimentation Equilibrium Experiments

A Beckman–Spinco Model E analytical ultracentrifuge equipped with both schlieren and Rayleigh interference optical systems was used in these experiments. The temperature was maintained at 20° for all experiments, and the velocity was regulated with an electronic speed control. Double-sector cells equipped with sapphire windows, a 12-mm Kel F-coated aluminum centerpiece and an interference mask were used for the equilibrium experiments. The amounts of solution, dialysate and bottom-forming liquid, FC-43, were measured with a micrometer syringe. For rotor velocities below 15,000 rpm, an An-J rotor was used, while at velocities above 15,000 rpm, either an An-D or An-H rotor was used. During equilibrium experiments, photographs were taken using both optical systems at about 12-hr intervals, but only interference pictures were used in the evaluation of the results. The photographic plates were measured with a Gaertner microcomparator.

Preparation of Native and Dissociated Complex for Equilibrium Experiments. The sample of the complex upon which sedimentation equilibrium experiments were performed was obtained from the fractions eluted from the final G-200 Sephadex column at the peak of activity and protein. The homogeneity of the sample was confirmed by the aforementioned techniques, and the solution (usually 1–2 ml of solution with about 3–6 mg/ml of protein) was dialyzed at least overnight against 250 ml of buffer D. All transfers of the solution and dialysate from this point were performed with care to prevent differential evaporation. The optical density of the solution was determined on an aliquot, and the concentration in milligrams of protein per milliliter was estimated. A portion was then separated for the equilibrium experiments on the native complex, and the remaining portion was utilized to prepare a sample of the dissociated complex.

About 1 ml of the solution of the native complex in buffer D was dialyzed at least 4 days at 5° against buffer G. The volume of the protein solution decreased with time of dialysis, but the approximate original volume was restored by addition of dialysate at the end of the dialysis time.

Measurement of Initial Concentration. To measure the initial concentration of the native complex or the dissociated complex (protein concentration, 1.5–3.0 mg/ml), an artificial boundary was formed in a capillary-type synthetic boundary cell. The solution channel of the cell was filled with 0.14 ml of the protein solution, and the reference channel was filled with 0.44 ml of dialysate. The boundary was formed at 5200 rpm, and after the formation of the boundary, the rotor velocity was reduced to 4000 rpm. Photographs were made periodically with both optical systems for about 2 hr. During this time the diffusing boundary extended neither to the bottom nor to the meniscus of the solution column, but the faster diffusing boundary of the initial salt gradient caused by differential evaporation between the solution and the dialysate during handling operations virtually disappeared. In some experiments the fringe count across the boundary was almost independent of time; but in others, particularly in solutions

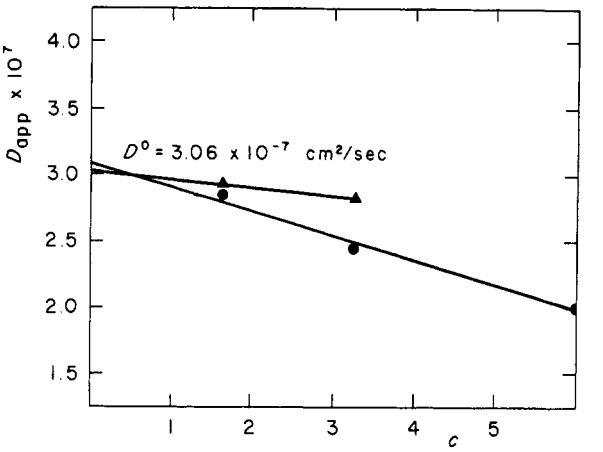

FIGURE 4: The dependence of the apparent diffusion coefficient of the complex upon concentration. The intercept, D^0, was taken as the average of the extrapolated data from sedimentation velocity (●) and synthetic boundary (▲) experiments. Units of D are cm^2/sec, and units of c are mg/ml.

of guanidinium chloride, the fringe count was carefully extrapolated to infinite time to obtain the correct value for the initial concentration of protein in fringes (Munk and Cox, 1972). Upon completion of the synthetic boundary experiment, the gradients in the cell were destroyed by shaking the rotor, and the base line was determined by photographing the fringe pattern after acceleration to 4000 rpm.

Alternatively, the initial concentration of the native complex was measured in a sedimentation experiment. The solution column of a double-sector cell was filled with 0.40 ml of protein solution, and an identical amount of dialysate was placed in the reference column. The rotor was accelerated to 52,000 rpm, and photographs were made periodically using both optical systems. The fringe count across the sedimenting boundary was determined and corrected for radial dilution, and after correction the fringe count was virtually constant. The base-line value was obtained from the photograph taken after complete sedimentation of the protein. The technique was not used for solutions in 6 M guanidinium chloride due to the unfavorable ratio of sedimentation to diffusion in this solution.

Low-Speed Equilibrium Experiments. The sedimentation equilibrium experiments were performed in an identical manner for the native and for the dissociated complex. In the bottom of each channel of a double-sector ultracentrifuge cell was placed about 0.05 ml of FC-43 bottom-forming liquid. The precise amount for matching of the menisci of the two channels in the cell was determined for each centerpiece. Enough protein solution (0.10 ml) was placed in the solution channel to produce a solution column of about 3 mm, and the reference channel was filled with 0.10 ml of dialysate. The interference patterns did not change with time after 30 hr at 4800 rpm (native complex) or 12,000 rpm (dissociated complex) although the experiments were usually continued to between 60 and 90 hr. This result showed that the systems were at sedimentation equilibrium; it also indicated that the protein did not undergo any gradual change during the experiment. For one native preparation, this latter point was checked in a different way. Before the equilibrium run, a sample was subjected to velocity sedimentation. The filled cell was laid aside at room temperature, and the velocity experiment was repeated when the equilibrium run had been completed about 4 days later. The velocity and

TABLE II: Average Amino Acid Composition of the Threonine-Sensitive Aspartokinase–Homoserine Dehydrogenase Complex.

Amino Acid	Residue/100 g of Protein[a]		*E. coli* K-12 Complex[h]		
	Av	±Std Dev	Nearest Integer/346,000	Calcd Wt % Composition	Nearest Integer/360,000
Lysine	4.89	0.12	132	4.71	132
Histidine	2.15	0.09	54	2.10	55
Arginine	7.64	0.28	169	8.13	187
Tryptophan[b,g]	0.77	0.08	14	0.62	12
Aspartic acid[c]	10.92	0.36	329	10.44	326
Threonine[d]	4.32		148	3.60	128
Serine[d]	5.58		222	5.45	225
Glutamic acid[c]	13.09	0.34	351	13.01	362
Proline[e]	3.22	0.09	115	4.32	160
Glycine	4.25	0.19	258	4.08	257
Alanine	7.23	0.08	352	7.28	368
Cysteine[e]	1.41	0.06	47	1.26	44
Valine[f]	6.82		238	7.34	266
Methionine	2.82	0.11	74	2.88	79
Isoleucine[f]	5.46		167	5.67	180
Leucine	11.16	0.19	342	10.77	342
Tyrosine[g]	3.38	0.12	72	3.27	72
Phenylalanine	4.90	0.15	115	5.08	124
Totals	100.01	2.26	3199	100.01	3319

[a] Four separate samples were analyzed using both oxidized and nonoxidized protein. [b] Samples 1 and 2 were not analyzed for tryptophan. [c] Free acid plus the amide form. [d] Corrected to zero hydrolysis time. Standard deviation not computed. [e] The content reflects the selection of values from oxidized protein only. Cysteine and cystine are analyzed as cysteic acid. [f] Corrected for incomplete hydrolysis. Standard deviation not computed. [g] The content reflects the selection of values from nonoxidized protein only. [h] Truffa-Bachi *et al.* (1968).

shape of the sedimenting boundaries were identical before and after the equilibrium run.

Base-line corrections for distortion of the optics and the cell window were made by measuring a water base line obtained by emptying the cell without dismantling it, rinsing it well with water, drying the cell under vacuum, refilling it with water, placing the water-filled cell back in the rotor, and accelerating the rotor to the speed of the experiment. Photographs taken at this speed provided the necessary information for the base-line corrections.

High-Speed Meniscus Depletion Experiments. For the meniscus depletion experiments, the initial concentration of the protein solution loaded into the cell was about 0.5 mg/ml, and the rotor speed was 9000–11,000 rpm for the native complex and 26,000 rpm for the dissociated complex. Procedures for loading the ultracentrifuge cell were the same as for the preceding experiment. High-speed experiments were continued for at least 48 hr, and a "water base line" was used for correction of optical and cell window distortions.

Evaluation of the Equilibrium Experiments. The data were evaluated by a program written for a CDC-6600 computer, and the results were analyzed according to the method of Munk and Cox (1972) using $\bar{M}_w$-type (log c *vs.* r^2), $\bar{M}_z$-type $[(1/r)(dc/dr)$ *vs.* $c]$, and M_{app}^{-1}-type $[M_{app}^{-1} \equiv (1 - \bar{v}_1\rho_0)\omega^2 cr/RT(dc/dr)$ *vs.* $c]$ plots. Here c is the concentration of protein in arbitrary units, r the distance from the rotational axis, ω the angular velocity, $\bar{v}_1$ the partial specific volume, ρ_0 the density of solvent, and RT has the usual meaning. For homogeneous, nonideal solutes the M_{app}^{-1} plot is linear in the first approximation; the intercept at zero concentration is the inverse molecular weight of the solute. The slope of the dependence is $B + (\bar{v}_1/M)$ for this case; we will neglect the term $\bar{v}_1/M$ subsequently. For the more general case involving heterogeneous, nonideal solutes, the dependence is not linear. However, the intercept at zero concentration of a tangent to the dependence at a point r will be $\bar{M}_z^r/(\bar{M}_w^r)^2$, and the slope of the tangent will be a complex function of both the second virial coefficient and terms dependent upon the heterogeneity. The latter terms in the slope may predominate at lower concentrations. Nevertheless, in cases where heterogeneity with respect to molecular weight is only moderate, the intercept of the least-squares line through the experimental points is not greatly different from the value of $\bar{M}_z/\bar{M}_w^2$ for the whole sample, although the slope of the line may be significantly reduced, and may in fact be negative. For homogeneous ideal solutes, the $\bar{M}_w$, $\bar{M}_z$, and $\bar{M}_w^2/\bar{M}_z$ obtained from all these treatments will be identical within experimental error. The extent of heterogeneity with respect to molecular weight and the influence of nonideality are difficult to evaluate from the primary $\bar{M}_w$- and $\bar{M}_z$-type plots; the third plot provides a sensitive indication of both heterogeneity and nonideality and requires no more data than those already obtained for the two primary plots (Munk and Cox, 1972).

Results

Purification and Homogeneity of the Complex. Fraction VI, obtained as outlined above and subsequently purified chromatographically through a G-200 Sephadex column for physical measurements, is apparently homogeneous as indicated by

TABLE III: Summary of Molecular Weight Determinations for the Threonine-Sensitive Aspartokinase–Homoserine Dehydrogenase Complex as Determined by Sedimentation Equilibrium.[a]

Sample	Method	$\bar{M}_w$[b]	$\bar{M}_z$	$(\bar{M}_w)^2/\bar{M}_z$[c]	$B \times 10^5$ [d]
3	Low Speed		347,000		
4	Low Speed		341,000		
5	Low Speed		361,000		
5	High Speed	326,000	328,000		
6	Low Speed	340,000	336,000	346,000	4.2
6	High Speed (11,000 rpm)	361,000	362,000		
6	High Speed (9,000 rpm)	354,000	358,000		
7	Low Speed	334,000	336,000	353,000	6.9
7	High Speed	338,000	358,000		
	Average	342,000	347,000	349,500	5.6
		M = 346,000 ± 15,000			

[a] The values summarize the information obtained from five different preparations of the complex. [b] The $\bar{M}_w$ for samples 3, 4, and 5 were not determined due to errors in c_0 measurements. [c] The values for $\bar{M}_w^2/\bar{M}_z$ and for the second virial coefficient were determined only for samples 6 and 7. [d] Units of B are (mole cm^3)/g^2.

analytical disc gels in the presence of threonine (Figure 1) and by sedimentation velocity experiments (Figure 2). Several different procedures have been used to measure the homoserine dehydrogenase activity of the enzyme complex isolated from different strains of *E. coli* (Truffa-Bachi *et al.*, 1968; Ogilvie *et al.*, 1969; Takahashi and Westhead, 1971). The assay procedures which have been used to determine specific activity of the homoserine dehydrogenase differ principally in the extinction coefficient used to measure the protein concentration, in the temperature used, and in the concentrations of potassium ion and of phosphate in the assay medium. When the complex isolated from strain 9723 was assayed by techniques comparable to those used in other laboratories, it was found to be at least as active as the complexes isolated from other strains. The aspartokinase (11.6 units/mg) from strain 9723 is nearly twice as active as the aspartokinase from strain K-12, but the difference is largely accounted for by the difference in temperature at which the assays were performed (Stadtman *et al.*, 1961).

Amino Acid Analysis. Table II shows the average of the amino acid compositions of four samples of the complex isolated from *E. coli* 9723, and compares the percentage composition of this complex with the composition calculated from that reported by Truffa-Bachi *et al.* (1968) for the complex from *E. coli* K-12. The amino acid compositions are, in general, very similar. There is a significant difference in the amino acid content of the two complexes only in two amino acid residues, threonine and proline. The difference in the proline values appears to be partly due to the selection of values from samples of oxidized protein in these experiments, since Matsubara and Sasaki (1969) report that the technique used here to recover tryptophan yields incorrect proline values. The percentage content of proline reported here compares favorably to the percentage content of proline from oxidized samples of the complex from strain K-12 (Truffa-Bachi *et al.*, 1968). Since threonine values require extrapolation to zero hydrolysis time to correct for loss of threonine during hydrolysis, it is difficult to compare results from any two analyses in different laboratories. However, sufficient data were obtained in these experiments to state that the threonine content of the complex from strain 9723 should not in any case be lower than the content reported. Somewhat higher values, in fact, appear more likely than lower values.

The total number of amino acid residues for the complex from strain 9723 (mol wt 346,000) is 3199 compared to 3319 for the similar complex from strain K-12 (mol wt 360,000; Truffa-Bachi *et al.*, 1968). The average residue weight is 108 for both complexes and the total difference in residues is 120. Therefore, the difference in the values for the molecular weights largely accounts for the difference in the nearest integer values for the amino acid residues.

Hydrodynamic Properties of the Native Complex. The standard sedimentation coefficient and the standard diffusion coefficient of the complex are, respectively, 11.4 S (Figure 3) and 3.13 × 10^{-7} cm^2/sec (Figure 4). The precision of the measurement of the diffusion coefficient is low due to the limited number of measurements of D_{app} and the uncertainty of the precise shape of the dependence of the diffusion coefficient upon concentration.

From the standard diffusion and sedimentation coefficients, the average value of the frictional coefficient of the complex, f = 1.32 × 10^{-7} g/sec, was calculated; and from it, the Stokes' radius, R_e = 69.8 A°, and the frictional ratio of the complex, f/f_{min} = 1.50, were obtained. Collectively, these results suggest that the native complex in solution is either rather elongated (ratio of the semiaxes, a/b = 9, no hydration) or highly hydrated (1.74 g of water/g of protein, spherical shape); or most likely, both quite elongated and quite highly hydrated (see Tanford, 1961, for the pertinent equations for these calculations). Preliminary electron micrographs (Maul, 1969) indicate that the complex may be rather elongated.

Molecular Weight of the Native Complex. The molecular weight of the native complex was estimated by the Svedberg method and by sedimentation equilibrium experiments using both the low- and the high-speed technique.

From the measured values of the sedimentation and diffusion coefficients, the molecular weight of the complex was calculated to be 334,000 ± 14,000. Because the precision of the measurement of the diffusion coefficients was low, this result was not heavily relied upon, but the value does agree quite well with the average value derived from sedimentation equilibrium experiments for the complex.

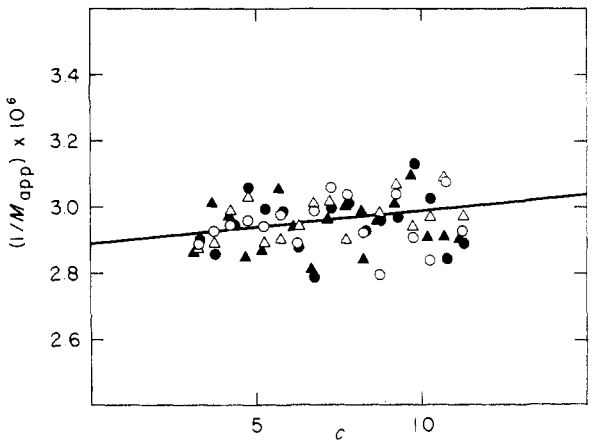

FIGURE 5: Low-speed sedimentation equilibrium. The inverse apparent molecular weight of the native complex as a function of concentration (sample 6, 1.64 mg/ml in buffer D, 4800 rpm). The data were obtained from interference photographs of the equilibrium distribution in the cell at: (△) 38.17 hr, (●) 45.51 hr, (○) 62.00 hr, and (▲) 62.02 hr. Units of c are fringes and units of r are cm. The molecular weight estimated from this plot was 346,000 and the second virial coefficient was 4.2×10^{-5} (mole cm^3)/g^2 (see Table III).

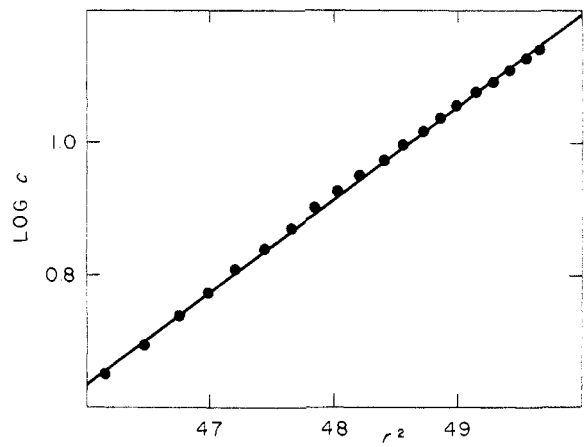

FIGURE 7: Low-speed sedimentation equilibrium. The apparent weight-average molecular weight of the dissociated complex in 6 M guanidinium chloride (sample 7, 3.0 mg/ml in buffer G, 12,000 rpm). The plot was obtained from an interference photograph of the equilibrium distribution in the cell at 64.05 hr. Units of c are fringes, and units of r are cm. The apparent $\bar{M}_w$ from the average slope of the dependence was 63,000.

Several low- and high-speed sedimentation equilibrium experiments were performed on the native complex. The results are summarized in Table III. The usual $\bar{M}_w$- and $\bar{M}_z$-type plots derived (not shown) from the low-speed experiments exhibited a relatively high degree of linearity. However, in the best preparations of the complex, the apparent $\bar{M}_z$ was less than the apparent $\bar{M}_w$. This suggests that even in dilute buffers, nonideal behavior of high molecular weight proteins may not be completely negligible. Figure 5 shows an M_{app}^{-1}-type plot (Munk and Cox, 1972) from a low-speed experiment on sample 6. The molecular weight of the solute derived from the intercept of the dependence at zero concentration is 346,000. The scatter of points seems large due to the expanded scale of the plot. Actually, the deviation of most points from the least-squares line is less than 5%. The slope of the line is the second virial coefficient, B (4.2×10^{-5} (mole cm^3)/g^2 in this experiment), and is theoretically exactly twice the quantity obtained from osmotic pressure experiments. A factor of 4100 was used to convert concentrations from fringes to g/cm^3 (Babul and Stellwagen, 1969).

The molecular weights derived from $\bar{M}_w$- and $\bar{M}_z$-type plots from the high-speed experiments were, in general, somewhat higher than the corresponding values obtained from the low-speed experiments. This is the expected result of the diminished influence of nonideality at the lower protein concentrations. M_{app}^{-1}-type plots were not constructed for high-speed experiments of the native complex. It should be noted that, in accordance with the usual practice (Yphantis, 1964), only those values of fringe displacement greater than 0.100 mm were utilized in the calculation of the least-squares fit of the data points in the high-speed experiments.

From both the low- and high-speed experiments, the average value of the molecular weight of the native complex was 346,000 ± 15,000.

Molecular Weight of the Subunits. Figure 6 shows the results from one of two polyacrylamide gel electrophoresis experiments in the presence of sodium dodecyl sulfate. The molecular weight of the subunit of the dissociated complex in this experiment was 88,600, while the average of the two experiments was 87,500. The uncertainty in this value from this limited number of experiments is estimated to be at least 10%. The standard curve is an unweighted least-squares fit of the data obtained from the protein standards. Upon completion of these experiments, it was learned that a molecular weight of 85,000 ± 3000 had been obtained elsewhere for the subunits in extensive experiments using this technique on the complex isolated from two strains of *E. coli* K-12 (Clark and Ogilvie, 1972; Dr. D. E. Wampler, personal communication).

The sedimentation equilibrium of the complex dissociated in 6 M guanidinium chloride is strongly influenced by nonideality. Nevertheless, the standard $\bar{M}_w$- and $\bar{M}_z$-type plots exhibit only marginal downward curvature (Figure 7) and yield very low values of molecular weight. For the low-speed experiment illustrated in Figure 7, the values of 63,000 and 52,600 were obtained for $\bar{M}_w$ and $\bar{M}_z$, respectively. It should be noted that the least-squares straight line in the $\bar{M}_z$-type

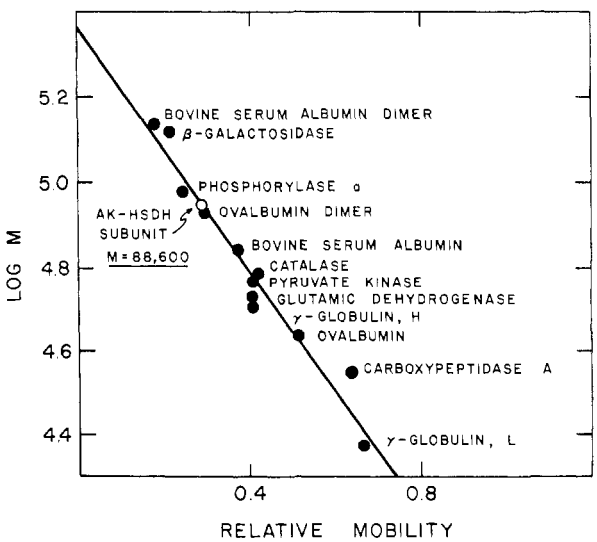

FIGURE 6: The molecular weight of the subunits of the complex as determined by gel electrophoresis in the presence of sodium dodecyl sulfate.

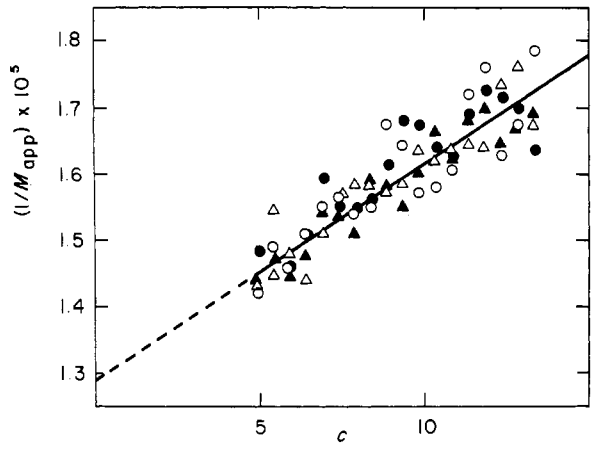

FIGURE 8: Low-speed sedimentation equilibrium. The inverse apparent molecular weight of the dissociated complex as a function of concentration. The same experiment as in Figure 7. The data were obtained from interference photographs of the equilibrium distribution in the cell at: (●) 64.05 hr, (○) 70.58 hr, (△) 88.30 hr, and (▲) 88.32 hr. Table IV gives the results obtained from this experiment. Units of concentration are fringes.

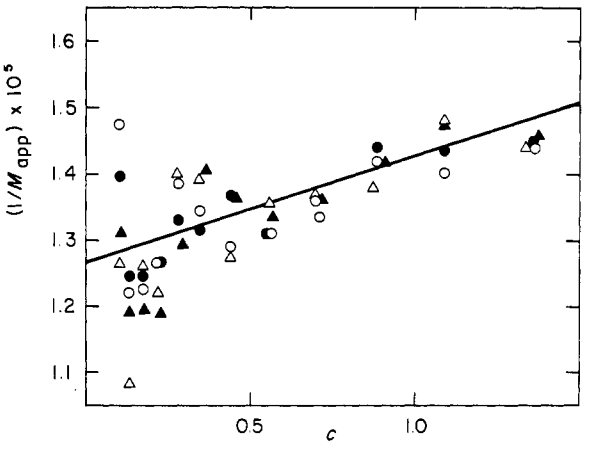

FIGURE 9: High-speed sedimentation equilibrium. The inverse apparent molecular weight of the subunits of the dissociated complex as a function of concentration (sample 7, 0.53 mg/ml in buffer G, 26,000 rpm). The data were obtained from interference photographs of the equilibrium distribution in the cell at: (●) 40.56 hr, (○) 55.08 hr, (▲) 66.08 hr, and (△) 66.10 hr. See Table IV for the results of this experiment. Units of concentration are mm of fringe displacement. One millimeter of fringe displacement equals approximately 3.64 fringes.

plot does not pass through the origin. For the high-speed experiments the apparent $\bar{M}_w$ and $\bar{M}_z$ values were higher but still considerably less than the values of $\bar{M}_w^2/\bar{M}_z$ obtained from M_{app}^{-1} plots.

Figures 8 and 9 illustrate M_{app}^{-1} plots for a low- and a high-speed experiment on the dissociated complex. The molecular weight averages $\bar{M}_w^2/\bar{M}_z$ and the apparent virial coefficients are presented in Table IV. A factor of 2870 was used to convert concentrations from fringes to g/cm³ (Munk and Cox, 1972); for our instrument, one fringe corresponds to a vertical fringe displacement of 0.275 mm. The average value of molecular weight is 80,000 ± 3000 for all experiments. If no reduction in $\bar{v}_1$ is employed, *i.e.*, $\bar{v}_1^* = \bar{v}_1$, then the molecular weight of the subunits would be 86,300. The values of the second virial coefficient are in the lower part of the range of values from the literature for denatured proteins in 6 M guanidinium chloride (Lapanje and Tanford, 1967; Castellino and Barker, 1968).

Discussion

The properties of the threonine-sensitive aspartokinase–homoserine hydrogenase complex from *E. coli* 9723 are summarized in Table V. These properties and the amino acid composition of the complex, when systematically compared with the properties for another complex having the same activities isolated from *E. coli* K-12 (Truffa-Bachi *et al.*, 1968), show little or no difference between the two complexes.

The weight-average and z-average molecular weights found for the complex from strain K-12 (357,000 and 350,000, respectively) lie high in the range of values reported here for the complex from strain 9723. The sedimentation coefficients of the two complexes are nearly identical, 11.4 and 11.5S. The analytical values for amino acid compositions differ but are similar, and the differences could result from variation in techniques. Considering the close relationship of the two organisms, one would expect the two strains to maintain a complex of identical or nearly identical molecular weight and shape but perhaps exhibiting minor differences in primary sequence and activities.

However, the molecular weight of the dissociated complex from strain 9723 in 6 M guanidinium chloride is close to 80,000, in contrast to the subunit molecular weight of 60,000 reported for the complex isolated from strain K-12. The present data for the complex from strain 9723 are most nearly compatible with a model composed of four subunits of similar or identical molecular weight rather than six. If the subunit molecular weight is multiplied by four, the predicted molecular weight for the complex is somewhat lower than the measured value. On the other hand, five or more subunits would yield a value that is much too high.

The chief deficiency of the present data is the assumption that $\bar{v}_1^*$ in concentrated guanidinium chloride is 0.01 ml/g less than the partial specific volume of the complex in dilute buffer. Although this assumption is consistent with the very limited data in the literature, it makes the calculated molecular weight somewhat uncertain. For example, if $\bar{v}_1^*$ is set equal to the $\bar{v}_1$ of the native complex, a molecular weight for the subunit which is precisely one-fourth of the measured value for the complex is obtained. The value of $\bar{v}_1^*$ could, of course, be manipulated arbitrarily to give molecular weights consistent

TABLE IV: Summary of the Molecular Weights Obtained by Sedimentation Equilibrium in 6 M Guanidinium Chloride.[a]

Sample	Method	$(\bar{M}_w)^2/\bar{M}_z$	$B \times 10^3$ [b]
3	Low speed	78,400	1.46
6	Low speed	79,900	0.92
6	High speed	84,700	1.32
7	Low speed	77,800	0.98
7	High speed	79,200	1.25
	Average	80,000 ± 3000	1.19 ± 0.25

[a] The values summarize the information obtained from three different preparations of the subunits in guanidinium chloride. The values for $(\bar{M}_w)^2/\bar{M}_z$ and the second virial coefficient, B, were derived from data plotted according to the method of Munk and Cox (1972) as in Figures 8 and 9. [b] The units of B are (mole cm³)/g².

TABLE V: Summary of Some Physical Properties of the Threonine-Sensitive Aspartokinase–Homoserine Dehydrogenase Complex and the Subunit Molecular Weight.[a]

Property	Method	Value Obtained
Molecular weight (M) of native complex	Sedimentation equilibrium	346,000 ± 15,000
	Svedberg equation	334,000 ± 14,000
Molecular weight (M^b) of dissociated complex	Sedimentation equilibrium in 6 M guanidinium chloride	80,000 ± 3000
	Sodium dodecyl sulfate gel electrophoresis	87,500
Second virial coefficient (B)	Munk and Cox (1972)	5.6×10^{-5} (mole cm^3)/g^2
Sedimentation coefficient, $s^0_{20,w}$	Sedimentation velocity	$11.4 \pm 0.1 \times 10^{-13}$ sec^{-1}
Diffusion coefficient, $D^0_{20,w}$	Sedimentation velocity and synthetic boundary	$3.13 \pm 0.10 \times 10^{-7}$ cm^2/sec
Frictional coefficient, f	$f = kT/D^0 = M(1 - \bar{v}_1\rho)/N_{av}s^0$	1.32×10^{-7} g/sec
Frictional ratio, f/f_{min}	$f/f_{min} = f/[6\pi\eta(3M\bar{v}_1/4\pi N_{av})^{1/3}]$	1.50
Stokes' radius, R_e	$R_e = f/6\pi\eta$	69.8 Å
Partial specific volume, $\bar{v}_1$	From amino acid content	0.735 cm^3/g

[a] The values reported in this table are either the experimental averages or have been calculated from the average value of an experimentally determined number without regard to the uncertainty in the number. [b] All other properties reported in this table refer to the native complex.

with any number of subunits. However, there is, as yet, no precedent in the literature for adjustments of $\bar{v}_1$* large enough to produce a model composed of three or five subunits. The difference between the subunit molecular weights obtained for the complex from the two strains of *E. coli* is not, in any case, explained by the uncertainty in $\bar{v}_1$*; this matter was treated in the same way in both cases.

The subunit composition of the complexes from the two strains may be different. However, the data obtained from gel electrophoresis in the presence of sodium dodecyl sulfate in this laboratory on the complex from strain 9723 and in two other laboratories on the complex from strain K-12 agree very well with a model of four subunits (Clark and Ogilvie, 1972). It seems, therefore, that the subunit composition of the complexes from the two strains is the same and that the neglect of nonideality in the earlier experiments may have produced low estimates of the molecular weight of the subunit from strain K-12.

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Sedimentation Equilibrium of Protein Solutions in Concentrated Guanidinium Chloride. Thermodynamic Nonideality and Protein Heterogeneity†

Petr Munk and David J. Cox

ABSTRACT: The theory of sedimentation equilibrium of heterogeneous nonideal solutes has been reviewed. It has been shown by simulation experiments that conventional treatments of experimental data neglecting the nonideality and heterogeneity may yield erroneous molecular weights, particularly when the low-speed technique is used. A simple procedure for the treatment of experimental data has been designed to eliminate these errors. The validity of the new procedure has been successfully tested on solutions of several proteins in 6 M guanidinium chloride. A density gradient is formed in complex solvents during centrifugation. The effect of this gradient on sedimentation equilibrium of macromolecular solutes has been studied in detail and has been shown to be negligible for protein solutions in guanidinium chloride. Differential evaporation from solution and solvent may produce large errors in measurements of solute concentration in a synthetic boundary cell. These errors can be detected and eliminated by simple modifications of the experimental technique.

Native proteins frequently consist of several polypeptide chains. Guanidinium chloride at high concentrations disrupts the noncovalent interactions that maintain the conformations of the individual chains and hold the chains together in the native structure. If a mild reducing agent is present, inter- and intrachain disulfide bonds are also broken, and the original aggregate is usually converted to a collection of separate chains, each of which exhibits the hydrodynamic and thermodynamic behavior of a statistical coil (Tanford *et al.*, 1967; Kawahara and Tanford, 1966; Lapanje and Tanford, 1967).

Sedimentation equilibrium experiments with proteins in a solution containing concentrated guanidinium chloride and a moderate amount of an appropriate mercaptan are used frequently for measurement of the average molecular weight of the subunits (Green and McKay, 1969; Truffa-Bachi *et al.*, 1969). These systems do, however, display some complexities which are frequently neglected in subunit studies because they are not commonly met in other contexts by protein chemists. Many native proteins behave nearly ideally at low concentration and the neglect of nonideality is not very damaging except in rather sophisticated studies of associating systems. On the other hand, the virial coefficients of proteins in guanidinium chloride are at least an order of magnitude higher than those of native globular proteins in normal solvents (Lapanje and Tanford, 1967; Castellino and Barker, 1968). If the effect of nonideality is ignored, erroneous conclusions may be drawn from sedimentation equilibrium measurements.

It is well known (Tanford, 1961) that the common treatments of sedimentation equilibrium data yield straight lines for ideal homogeneous solutes and curved plots for nonideal as well as heterogeneous samples. Experiments that produce reasonably straight lines may be described as indicating that the samples concerned are homogeneous within the limits of the technique. It is, however, not usually clear how broad are the limits of the technique. Nonideality tends to mask curvature due to heterogeneity. In dealing with a protein composed of subunits, it is important to ask whether sedimentation equilibrium in concentrated guanidinium chloride can be expected to produce any useful information bearing on the identity or nonidentity of subunits.

A sedimentation equilibrium experiment yields apparent molecular weights over a range of solute concentrations, and, for a homogeneous solute, a single experiment can, in principle, provide the information needed to eliminate the effect of nonideality. The problem may be approached by plotting the apparent molecular weight against concentration and obtaining the true value by extrapolation to infinite dilution (Seery *et al.*, 1967, 1970). If such a procedure is to be of practical use, it must not demand data more precise than those provided by real experiments. Experimental errors that tend to produce spurious curvature in the data plots must be eliminated. It is also necessary to know which average of molecular weight is produced by any given extrapolation procedure if the solute happens to be heterogeneous. Alternatively, one of the "ideal" molecular weight moments can be calculated point by point along the solution column (Roark and Yphantis, 1969). If the solute is homogeneous, the ideal moments are essentially independent of concentration, and the values ob-

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