Biosynthesis of Urea

XIV. THE QUATERNARY STRUCTURE OF ARGININOSUCCINASE*

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SUMMARY

Argininosuccinase from bovine liver with a molecular weight of 202,000 completely dissociates at pH 9 or 10 to 5.6 S subunits. The catalytically active tetramer can be reconstituted by bringing the pH back to 7.5. Determined by sedimentation equilibrium the subunit molecular weight is 100,000 ± 2,500, corresponding to that of a dimer. Sedimentation velocity experiments show that initially the dissociated preparation consists of primarily dimer. Polymerization, apparently through the participation of dimers, is observed after long periods as sedimentation equilibrium analyses show the presence of small amounts of tetramer not in chemical equilibrium with the dimer, and some hexamer.

In concentrated solutions of urea or guanidine hydrochloride, the molecular weight by sedimentation equilibrium is 50,400 ± 1,100. A similar value was estimated by gel electrophoresis in sodium dodecyl sulfate in the absence of reducing agent. The four polypeptide chains are, therefore, identical in size. The enzyme is devoid of disulfide bonds; in guanidine hydrochloride 16 moles of free sulfhydryl groups were determined with 5,5'-dithiobis(2-nitrobenzoate), and this value is equal to the number of half-cystine residues per mole of protein. End group analyses showed that the carboxyl-terminal amino acid is glutamine, the penultimate residue is leucine, and there are four identical terminal sequences per mole. Cyanogen bromide cleavage produced 13 to 14 peptides, one-fourth of the expected number of fragments based on a methionine content of 51 residues per mole of protein. In accord with these results, tryptic peptide patterns showed only one-fourth of the total number of peptides predicted from the lysine and arginine content of the enzyme. The number of peptides containing several other specific amino acid residues was also consistent with the number predicted from an average distribution of residues among four polypeptide chains. These data show that argininosuccinase is composed of four subunit polypeptide chains that are apparently identical, or very similar, in amino acid sequence.

The distribution of the 16 sulfhydryl groups was related to quaternary structure from studies with sulfhydryl reagents. In the active tetramer, four sulfhydryl groups are readily accessible; presumably, they are located on or near the surface of the enzyme and are remote from the active sites, as disulfide or mercaptide formation with sulfhydryl reagents does not affect catalytic activity. A second group of four is masked within the regions of bonding between dimeric subunits; they are released concomitantly with dissociation of the tetramer to dimers. The remaining eight sulfhydryl groups only become accessible as the dissociation of dimers to monomers proceeds in the presence of strong dissociating agents. From the results of dissociation behavior and sulfhydryl reactivity, at least two modes of subunit interactions can be distinguished.

Electron micrographs of negatively stained argininosuccinase support this four-subunit model. The model is discussed with respect to modes of dissociation, geometric arrangement, and symmetry.

Argininosuccinase, molecular weight 202,000, dissociates at low temperatures to catalytically inactive subunits with an estimated molecular weight of 100,000 (1, 2). The presence of the substrates, argininosuccinate and arginine, in low concentrations can counteract dissociation. Phosphate and sulfate also exert a protective effect, but are required in much higher concentrations. Temperature-dependent equilibria between the two forms of the enzyme were described in our last publication (2). From the negative temperature dependence and the large positive change in entropy, 189 e.u., it was concluded that the active enzyme was stabilized mainly by hydrophobic bonds. The present study was undertaken to elucidate the oligomeric structure of the enzyme and the subunit interactions that determine the quaternary structure.

EXPERIMENTAL PROCEDURE

Materials—Argininosuccinic acid, synthesized enzymatically, was isolated as the barium salt, converted to the potassium salt, and assayed (1). Reagents were obtained from the following sources: "ultrapure" urea and guanidine hydrochloride from Mann Research Laboratories; [1-14C]iodoacetic acid from

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New England Nuclear; cyanogen bromide from Matheson, Coleman, and Bell, East Rutherford, N. J.; the sodium salt of p-hydroxymercuribenzoic acid from Sigma; dithiothreitol from Calbiochem; SDS\(^2\) and 2 mercaptoethanol from Pierce Chemical Co., Rockford, Ill.; “Distilled in Glass Solvents” for chromatography and electrophoresis from Burdick and Jackson, Muskegan, Mich. Trypsin (TPCK-treated) and DFP-carboxypeptidases A and B were purchased from Worthington.

Dialysis tubing was boiled for 30 min in 0.1 M KHCO\(_3\) containing 1 mM EDTA, then washed and stored in 1 mM EDTA, to remove traces of ultraviolet-absorbing material and metal ions; before use it was thoroughly rinsed with distilled water.

Preparation of Argininosuccinase—Argininosuccinase from steer liver was prepared and assayed according to the usual procedures of this laboratory (1, 2). Twice crystallized material with a specific activity of 1300 to 1400 amoles of substrate cleaved per hour per mg was used throughout these studies. Protein was determined from the absorbance at 280 nm. In 0.05 M potassium phosphate, pH 7.5, the extinction coefficient, \(E_{280}\), of crystalline argininosuccinase is 1.298; this value is based on the amino acid content of a sample of known absorbance (4).

**SDS-Polyacrylamide Gel Electrophoresis**—The SDS-polypeptide complexes were prepared according to the procedure of Weber and Osborn (5) except that the concentration of SDS and mercaptoethanol was 0.1 and 1%, respectively. Argininosuccinase was dissociated in SDS both in the presence and in the absence of mercaptoethanol. The gels contained 7.5% acrylamide, 0.2% cross-linker, 0.1 M sodium phosphate, pH 7.0, and 0.1% SDS. Electrophoresis was conducted at 7 volts per cm until the tracking dye was 1 to 2 cm from the bottom of the tube. The gels were removed and a small piece of Nichrome wire was inserted into the gel to mark the position of the dye; the gels were then stained in freshly prepared solutions of 0.25% Coomassie blue or 1% Amido black for several hours and destained in 7% acetic acid.

The reference proteins and their subunit or polypeptide molecular weights were: bovine thyroglobulin, 160,000; bovine serum albumin, 68,000; ovalbumin, 45,000; carboxypeptidase A, 34,300; trypsin, 23,800; and chymotrypsin, 13,000 and 11,000. The molecular weights were taken from the reports of Weber and Osborn (6) and Tanford (6).

**Ultracentrifuge Studies**—Sedimentation velocity and sedimentation equilibrium runs were made in a Spinco model E analytical ultracentrifuge equipped with a phase plate schlieren diaphragm, ultraviolet optics and an automatic scanner, and a rotor temperature indicator control unit.

Determinations of molecular weights by sedimentation equilibrium were made at several protein concentrations at 5 or 20° with 3-mm liquid columns in a 12-mm, three-channel centerpiece according to the high speed equilibrium method of Yphantis (7).

For the determination of the molecular weight of the dimer, the composition of the dissociating buffer was 0.1 M glycine, 2 mM EDTA, and 1 mM dithiothreitol adjusted at 4° to pH 10 with KOH. A stock protein solution was prepared, about 1 mg per ml, close to the highest protein concentration used in a series of experiments. This solution of enzyme was equilibrated with the dissociating buffer by dialysis (8) at 4° for 10 hours to ensure equilibrium with the dialysis buffer. The solutions for sedimentation analysis were prepared by appropriate dilution with the dialysate. The sedimentation equilibrium runs were carried out at 18,000 rpm (\(c = 4\)) at 5° with initial protein concentrations of 0.01, 0.03, and 0.07 g per dl.

Equilibrium was attained after 18 hours at 18,000 rpm, ascertained by observing the fringe pattern during the next 6 hours. Fringe displacements, the average of three white and two black fringes, were measured on a Nikon model C micro-comparator. Blank corrections were obtained from the first photograph taken after acceleration to 5000 rpm. Least squares analysis of the data showed a marked increase in molecular weight as the protein concentration decreased. Therefore, points were read through the cell at intervals of 700 \(\mu\)m until the \(y\)-coordinate readings changed by 10 \(\mu\)m, then every 50 or 100 \(\mu\)m until fringes could be longer be resolved at the base of the cell. These data were processed in a CDC 6600 computer with the FORTRAN IV program, developed by Roark and Yphantis for which we are very much indebted to Dr. D. A. Yphantis. The computer program provides the number-, weight-, and \(z\)-average molecular weight at each point in the cell, as well as a series of charge-independent moments (9, 10). The point-average molecular weights \(M_1(r)\) are expressed in terms of \(\sigma\), the effective reduced molecular weight, defined by Yphantis (7); the values of \(\sigma_1(r)\) correspond to the various molecular weight averages at the points \(r\) according to the general expression \(M_{app} = \sigma \times \left[RT/\omega^2(1 - \rho\sigma)\right]\). For the given experimental conditions the expression in brackets is a constant having the value of 25,035.

For the determination of the molecular weight of argininosuccinase in guanidine hydrochloride, at 20°, absorbance scans were made at 280 nm as described by Schachman and Edelstein (11). An initial scan was made after acceleration to 5,000 rpm, and duplicate scans were taken at 36,000 rpm after 6, 24, 28, and 32 hours at which time sedimentation equilibrium had been achieved. After overspeeding to 44,000 rpm and maintaining this speed for 6 hours, a final scan was made to ascertain the absorbance baseline at the meniscus.

Weight-average molecular weights for the enzyme in 6 M guanidine hydrochloride were calculated from least squares analyses\(^3\) of the slope from plots of In concentration versus radius\(^2\). Concentration was expressed as absorbance at 280 nm measured directly from scanner traces.

The samples were thoroughly equilibrated with the reference solutions by exhaustive dialysis (18 to 60 hours). The viscousity and the density of the solvents were determined experimentally or calculated according to the method of Kawahara and Tanford (12). The partial specific volume of argininosuccinase is 0.734 (4).

**Carboxymethylation of Argininosuccinase**—Carboxymethylation was conducted essentially according to the procedure of Crestfield et al. (13). Based on the amount of carboxymethyl-cysteine determined by amino acid analysis, 15 to 16 residues of cysteine per mole of protein had reacted with iodoacetate; small amounts (less than 5%) of the carboxymethyl derivatives of histidine and methionine were also observed. Labeled carboxymethyl argininosuccinase was prepared with [\(^14\)C]iodoacetic acid and the reaction products were analyzed in the same way. The specific radioactivity of these preparations was 700 to 1000 dpm per nmole of cysteine.

\(^2\) The abbreviations used are: SDS, sodium dodecyl sulfate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; DFP, diisopropylphosphoryl-; DNS, dimethylaminonaphthalene-sulfonyl-; p-HMB, p-hydroxymercuribenzoate; DTNB, 5,5’-dithiobis (2-nitrobenzoate); TNB, thionitrobenzoate anion.

\(^3\) The sedimentation equilibrium runs were carried out in the laboratory of Dr. R. C. Warner, and the data were processed with a computer using a least squares analysis program written in the BASIC system. We are indebted to Dr. Warner for his critical evaluation of the data and for the use of these facilities.
Amino Acid Analysis—Samples were hydrolyzed in constant boiling HCl (5.7 M) at 110° for 22 hours in carefully evacuated and sealed Pyrex tubes. The hydrolysates were evaporated twice in a rotary evaporator at 50° to remove HCl, dissolved in 0.2 M citrate buffer, pH 2.2, and analyzed according to the procedure of Spackman et al. (14) with the accelerated system in a Jelco model JLC-5AH automatic amino acid analyzer equipped with a programmer, a photocell with a 6-mm optical light path, and a high sensitivity recorder (4 to 5 mV).

Determination of COOH-terminal Amino Acids—Hydrazinolysis was carried out according to the method of Akabori et al. (15). After removal of the hydrazides by extraction with isovalerianolde-hyde or by fractionation on an Amberlite IRC-50 column (16), the small amounts of free amino acids found were determined by automated amino acid analysis.

Argininosuccinase was carboxymethylated as described earlier, or maleylated by treatment with maleic anhydride by a modification of the method described by Butler et al. (17).

Experiments with DFP-carboxypeptidases A and B were conducted essentially according to the methods described by Ambler (18). The optimal conditions for peptidase hydrolysis were determined from a series of preliminary experiments. In all of the experiments, the concentration of argininosuccinase was based on the amino acid content of an aliquot subjected to acid hydrolysis. The amino acids released by carboxypeptidase were quantitated on the automatic amino acid analyzer. In the kinetic study, Experiment 2, the 14-hour sample was deproteinized by the resin bead method after the addition of norleucine as internal standard.

The DNS-derivatives of the carboxypeptidase A digestion products were obtained by dissolving the lyophilized incubation mixture in 0.1 ml of 0.2 M NaHCO₃ and treating this solution with DNS-chloride according to the method of Hartley (19). After acetylation, the free DNS-amino acids were obtained by extraction of the reaction mixture with ethyl acetate; the ethyl acetate was removed, and the residue was dissolved in 50% pyridine. The DNS-amino acids were separated by thin layer chromatography on double coated polyamide sheets using the solvent systems of Woods and Wang (20) and Crowshaw et al. (21) and were identified from the position of known DNS-amino acids on the reverse side of the sheets.

Cyanogen Bromide Cleavage—A lyophilized preparation of [1-¹⁴C]cyanogenmethylargininosuccinase was dissolved in 70% formic acid at a concentration of 5 to 10 mg per ml. Cyanogen bromide was added in 50-fold molar excess of the total number of methionyl residues; the tube was tightly stoppered, and the reaction was allowed to proceed at room temperature for 24 hours according to the method of Steers et al. (22). After cleavage the reaction mixture was diluted 5-fold with water and lyophilized twice.

The products were separated by high voltage paper electrophoresis on strips of Whatman No. 3MM paper. Approximately 1 mg of material, dissolved in 0.02 ml of 70% formic acid or 50% acetic acid, was applied to the paper, taking care that the sample should not dry completely. After electrophoresis in pyridine-acetic acid-H₂O (1:10:89), pH 3.5, under Varsol at 5000 volts for 120 min, the paper strip was air-dried and dipped in 0.3% acetic acid-H₂O (1:10:89), pH 3.5, under Varsol at 5000 volts for 30 min to allow complete conversion of the carboxyl-terminal residues either to homoserine or to its lactone form. Samples of 50, 100, and 200 µg were applied to the gels, and electrophoresis was carried out at 3 ma per tube until the tracking dye had migrated to within 1 cm of the end. The gels were stained with 1% Amido black or 0.25% Coomassie blue and destained in 7% acetic acid.

Peptide Maps of Tryptic Digests—Approximately 10 mg of [¹⁴C]labeled cyanogenmethylated argininosuccinase in 2 ml of 0.2 at NH₄HCO₃, pH 8.0, were digested with 100 µg of TPCK-trypsin at 37°. After 1 hour a second addition of 100 µg of TPCK-trypsin was made, and the digestion allowed to proceed for 8 to 16 hours. Small amounts of insoluble material if present were removed by centrifugation and the sample was shell-frozen and lyophilized. The dry material was easily soluble in water or dilute ammonia.

Peptide maps of the tryptic hydrolysates were made according to standard techniques (25). Samples of the tryptic peptides (1 to 2 mg) were applied to Whatman No. 3MM paper and were separated by descending chromatography in 1-butanol-acetic acid-H₂O (4:1:5) for 18 hours followed in the second dimension by electrophoresis in pyridine-acetic acid-H₂O (1:10:89), pH 3.6, under Varsol at 2400 volts for 90 min. Radioautographs were made by overlaying the peptide maps with Kodak No-Screen 54-T paper.

Titration of Sulphydryl Groups—Thiol groups were determined by titration with p-HMB according to the spectrophotometric method of Boyer (26). The concentration of p-HMB in a stock solution was measured spectrophotometrically at pH 7 with a molar extinction coefficient at 232 nm of 1.69 x 10⁴; the purity was estimated to be >98% on the basis of titration with glutathione. Argininosuccinase (0.3 to 1 mg) in 0.50 ml of 0.01 M potassium phosphate, pH 7.0, was titrated with small additions (10 µl) of a standard solution of p-HMB (1 x 10⁻³ M in 0.01 M potassium phosphate, pH 7.0) by following the change in absorbance at 250 nm. The equivalence point was determined graphically after correction of the data for dilution and for the absorbance of p-HMB and of protein measured in separate cuvettes. The rate of reaction with p-HMB was extremely rapid; the absorbance at 250 nm increased to its maximum immediately after each addition of reagent throughout the course of the titration.

Sulphydryl determinations with Ellman's reagent, DTNB, were made essentially as described by Janatova et al. (27), except that the total volume was 0.40 ml. Absorbance at 412 nm was measured in a Gifford recording spectrophotometer equipped with a thermostatically maintained cell chamber or in a Zeiss spectrophotometer. Aliquots (10 to 50 µl) of a solution of argininosuccinase of known protein concentration (between 10 and 200 µg per ml) were added to cuvettes that contained 0.29 to 0.25 ml of 0.0074 M potassium phosphate, pH 8.1, 0.02 ml of 0.04 M EDTA, and 0.08 ml of 0.01 M DTNB. The solution was mixed thoroughly and read immediately against a blank without enzyme. If the final pH in the cuvette was 7.0 to 7.6, there was no drift in the reagent blank. Denaturing agents when present by polyacrylamide gel electrophoresis in two systems: the first was that described by Davis (23); 6.7% gels in Tris glycine buffer, pH 9.1, contained 8 M urea. The second system was described by Lin and Tadayon (24); 7% polyacrylamide gels contained 5 M urea and 0.25% Triton, and electrophoresis was conducted with a discontinuous buffer system, running pH 2.7. The lyophilized cyanogen bromide fragments were dissolved in urea and the appropriate buffer and were incubated at 37° for 30 min to allow complete conversion of the carboxyl-terminal residues either to homoserine or to its lactone form. Samples of 50, 100, and 200 µg were applied to the gels, and electrophoresis was carried out at 3 ma per tube until the tracking dye had migrated to within 1 cm of the end. The gels were stained with 1% Amido black or 0.25% Coomassie blue and destained in 7% acetic acid.

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were added with the buffer, the concentrations are given in the appropriate table. Freshly prepared reduced glutathione was used as a standard in each set of experiments; a millimolar extinction coefficient of 13.6 was obtained for the thionitrobenzozate anion, in agreement with the value determined by Ellman (28). All sulfhydryl determinations were performed in duplicate at two concentrations of protein. Samples (50 μl) from the DTNB-containing reaction mixture could be assayed directly for argininosuccinase activity.

RESULTS

Oligomeric Structure of Argininosuccinase

Sedimentation Velocity Studies on 5.6 S Subunits—In our earlier study on the dissociation of enzymatically active argininosuccinase in the cold, it was found that after long incubation of the native enzyme at pH 8.3 in imidazole buffer at 0°, three species could be separated by sucrose gradient sedimentation. About 70% of the protein was present in the 5.6 S peak, about 13% in a region corresponding to 9.3 S (the active enzyme), and the remaining amount in the region corresponding to 15 S (2).

In order to determine the true molecular weight and composition of the dissociation products, an attempt was made to define conditions under which the enzyme would dissociate nearly quantitatively to the 5.6 S form. Because increases in pH increase the rate of dissociation and affect the equilibrium between the two species in favor of dissociation, more alkaline conditions were chosen. At pH 10 in glycine buffer, the enzyme was completely inactivated within 30 min at 0° and more slowly at room temperature, and the loss of activity was reversible. Even after several hours about 90% of the original specific activity was restored when the enzyme was reactivated by dialysis at 4° against 0.1 M glycine buffer, pH 7.5, containing 5 mM 2-mercaptoethanol followed by incubation at 38°. The pH-dependent dissociation appears to be similar to cold-induced dissociation. Whether the enzyme is inactivated at 0° or at room temperature by alkaline pH, sedimentation velocity experiments showed that approximately 90% of the protein sedimented as a symmetrical peak at 5.6 S, and the remaining 10% was located under a more rapidly sedimenting boundary (Fig. 1A). The proportion of this high molecular weight material increased with time, more rapidly at 23° than at 4°. After 30 hours at 4° it comprised approximately one-third of the total protein, sedimenting at about 13 to 14 S.

Presumably, the 5.6 S subunits with newly exposed bonding areas tend to form aggregates and this effect is accentuated by an increase in alkalinity. The tendency to aggregate was greatly reduced if the enzyme was dissociated in the presence of 2 mM EDTA and 1 to 2 mM dithiothreitol or mercaptoethanol. As shown in the sedimentation patterns in Fig. 1B, the enzyme appears to be almost completely dissociated. A single peak corresponding to 5.5 S was observed, even after the samples were held at 0° for 24 hours. The degree of boundary spreading at the leading edge varied somewhat between experiments and may be indicative of some association.

Molecular Weight of 5.6 S Subunits—Argininosuccinase, 1.2 mg per ml, was dissociated at pH 10, and a preliminary sedimentation velocity run showed that dissociation was complete; a single peak sedimenting at 5.6 S was observed, and there was no component smaller than 5.6 S in the solution. High speed equilibrium runs were carried out, as described under “Experimental Procedure,” at initial concentrations of 0.01, 0.03, and 0.07 g per dl to ascertain the molecular weight of the dimeric subunit. The logarithm of the net fringe displacement as a function of the comparator x-coordinate is shown in Fig. 2 for two protein concentrations at sedimentation equilibrium. The departure from linearity with decrease in concentration is clearly evident; the discrepancy between the points and the solid line indicates heterogeneity.

The point-average molecular weight moments were computed for every point in the cell for each protein concentration, and the data were analyzed according to the method of Yphantis (7) in order to define the system in terms of concentration dependence and dispersity. If the system is a single thermodynamic component such as a rapidly established dimer-tetramer equilibrium,
then the various point molecular weight averages at the three different initial concentrations will be identical functions only of concentration $c(r)$, not of position. On the other hand, the molecular weight averages of a purely heterogeneous solute will be a function only of position, assuming fractionation of the solute during the run.

The values of $\sigma_w(r)$ were plotted as a function of concentration, and a different curve was obtained for each initial concentration. But when these same values of $\sigma_w(r)$ were plotted against $b^2 - r^2$, where $b$ represents the distance from the axis of rotation to the base of the cell, most of the experimental values at the three different initial concentrations fell on one continuous curve (Fig. 3). The data show that the molecular weight averages are a function of position rather than of concentration. Therefore the system is primarily heterogeneous, and chemical equilibrium between the species is not attained during the time of centrifugation. In these experiments the most likely cause of heterogeneity is the presence of oligomers not in rapid equilibrium with the subunits.

In Fig. 3 there is some deviation of $\sigma_w(r)$ from the curve at the maximum observed fringe displacements. At the lowest initial concentration, readings were taken to within 0.05 mm of the bottom of the cell, and the base meniscus was normal. However, in the two higher concentration channels the fringes were not reached in the region near the bottom of the cell. As evidenced by the thickened base menisci and schlieren pattern, significant fractionation had occurred during the run, which in effect removed a portion of the heavy component from the observable concentration distribution. Consequently, the data from the two higher concentration channels can be used to define the smallest species, while the lowest concentration channel provides the molecular weight distribution of the entire solution (as initially loaded) and some information about the interfering polymers.

The molecular weight of the 5.6 S subunit was evaluated by extrapolation of the point-average molecular weights to zero concentration, from the quantity $2M_k(r) - M_w(r)$, and from monomer-$n$-mer plots (9, 29). The various molecular weight averages are presented in Fig. 4 as functions of $c(r)$ for each initial concentration. The downward trend of the values of $\sigma_w(r)$ may indicate some nonideality, but this should not significantly affect the results since $\sigma_w$ and $\sigma_v$ do not decrease, and the concentrations used in these experiments were all under 0.1%.

Using only the data from the two higher concentration channels where the 5.6 S subunit is in excess, extrapolation of $\sigma_w$, $\sigma_o$, and $\sigma_z$ to zero concentration afforded a value for $\sigma_1$ of $4.0 \pm 0.1 \text{ cm}^{-2}$, which corresponds to a molecular weight of $100,000 \pm 2,500$.

Shown also in Fig. 4, the values for the subunit molecular weight calculated from the quantity $2\sigma_w - \sigma_o$ (7) fall on a straight line at $\sigma_1 = 4.0 \pm 0.2 \text{ cm}^{-2}$ throughout the major portion of the concentration gradient. Thus, the results of these two methods are in accord; with a molecular weight of 100,000 the 5.6 S species corresponds to a dimer.

The data presented in Fig. 4 were also examined in terms of the relationship $M_k = -nM_1^2/(M_k - 1) + (n + 1)M_1$, which has been shown to be true for any system consisting of two ideal sedimenting species related in molecular weight by $n$, whether or not the species are in chemical equilibrium (9, 29). $M_k$ corresponds to the number-average, weight-average, $z$-average, and $z + 1$-average molecular weight as $k = 0, 1, 2,$ and 3, respectively. With a solution comprised of any combination of the two species, when $M_k$ is plotted versus $1/M_k - 1$ all of the experimental values will fall on a straight line with a slope of $-nM_1^2$ and with an intercept of $(n + 1)M_1$. Several such two-component combinations are depicted in Fig. 5, calculated on the basis of a molecular weight of 100,000 for $M_1$ represented as $A_1$ in the figure. Inspection of Fig. 5 shows that at the two higher initial concentrations the experimentally determined molecular weight averages $M_k(r)$ fell just above the predicted dimer-tetramer line. The values of $M_k(r)$ shown in the figure suggest that the extent of association is limited, the dimer appears to be the predominant species together with some tetramer. At
the lowest initial concentration however the experimental points coincided with those of the other concentration channels only in the initial part of the concentration gradient, where dimer and tetramer appear to be present. Thereafter, the upward deviation is marked, indicating the presence of a component heavier than tetramer, and the data suggest that this component may be a hexamer. As mentioned earlier, differences between the three different concentration channels are to be expected since the lowest initial concentration represents the average molecular weight distribution of the entire solution, but in the two higher concentration channels fractionation has reduced the contribution of the heavy components. The molecular weight of the subunit was calculated from the slope and the intercept of the line defined by these experimental values and an average value of 103,406 was obtained, in agreement with that evaluated by extrapolation to zero concentration and from the quantity $2M_c(r) - M_a(r)$.

There is no indication that dissociation proceeds to a monomeric subunit of molecular weight 50,000. The presence of a monomer would cause divergence of the points toward $A_1$ along the monomer-dimer line indicated in Fig. 5.

These studies with argininosuccinase, sedimentation velocity and sedimentation equilibrium, indicate that dissociation at low temperatures and alkaline pH initially involves the conversion of tetramer to predominantly dimer. Prolonged exposure to dissociating conditions may result in secondary changes in the dimeric subunit such that the dimer associates to polymers of higher than tetramer. From the sedimentation equilibrium data, the preparation appears to contain small amounts of tetramer not in chemical equilibrium with the dimer, and a significant amount of hexamer.

**Molecular Weight after Dissociation in SDS**—To obtain a preliminary estimate of the size of the polypeptide chains of argininosuccinase, preparations of the enzyme were dissociated with SDS and mercaptoethanol and the molecular weight was estimated by the method of SDS-polyacrylamide gel electrophoresis (5, 30, 31). Argininosuccinase and carboxymethylated argininosuccinase were prepared for electrophoresis as described under “Experimental Procedure.” Polyacrylamide gel columns were calibrated by determining the mobilities of the SDS complexes of several known proteins. The relationship between electrophoretic mobility and polypeptide molecular weight is illustrated in Fig. 6A. The mobility of the SDS-argininosuccinase complex, determined in the same run, corresponded to a molecular weight of 54,000. The results of several experiments varied between 50,000 and 55,000, whether or not mercaptoethanol was present. Both untreated and carboxymethylated preparations of argininosuccinase migrated at the same rate as a single narrow band (Fig. 6B), indicating that the polypeptide chains are of identical molecular weight. This was the first evidence that argininosuccinase can be dissociated into subunits smaller than those found after dissociation in the cold.

**Molecular Weight after Dissociation in 6 M Guanidine Hydrochloride**—Dissociation of argininosuccinase to a smaller subunit was also observed in 6 M guanidine hydrochloride and 8 M urea. Sedimentation velocity studies with a reduced, carboxymethylated preparation of the enzyme, 2.7 mg per ml in 6 M guanidine hydrochloride, showed a single, symmetrical peak sedimenting with an S value of 1.95. This is presumably the same subunit species found after dissociation with SDS and represents the randomly coiled polypeptide chain.

The molecular weight was determined with the high speed equilibrium method of Yphantis (7) as described under “Experimental Procedure.” Argininosuccinase, dissolved in 6 M guanidine hydrochloride, 0.05 M potassium phosphate, and 2 mM EDTA, pH 7.5, was equilibrated against this same reference

**Fig. 5.** Monomer-n-mer plot for dissociated (5.6 S) argininosuccinase. The experimental values are from the equilibrium experiment presented in Fig. 4. The circles are values for an initial concentration of 0.07 g per dl; $\sigma_0$ versus $1/\sigma_0$, $\sigma_1$ versus $1/\sigma_1$, $\sigma_2$ versus $1/\sigma_2$, $\sigma_3$ versus $1/\sigma_3$, $\sigma_4$ versus $1/\sigma_4$, $\sigma_5$ versus $1/\sigma_5$, $\sigma_6$ versus $1/\sigma_6$, $\sigma_7$ versus $1/\sigma_7$, $\sigma_8$ versus $1/\sigma_8$, $\sigma_9$ versus $1/\sigma_9$, $\sigma_{10}$ versus $1/\sigma_{10}$. The triangles are values for an initial concentration of 0.03 g per dl; $\sigma_0$ versus $1/\sigma_0$, $\sigma_1$ versus $1/\sigma_1$, $\sigma_2$ versus $1/\sigma_2$, $\sigma_3$ versus $1/\sigma_3$, $\sigma_4$ versus $1/\sigma_4$, $\sigma_5$ versus $1/\sigma_5$, $\sigma_6$ versus $1/\sigma_6$, $\sigma_7$ versus $1/\sigma_7$, $\sigma_8$ versus $1/\sigma_8$, $\sigma_9$ versus $1/\sigma_9$, $\sigma_{10}$ versus $1/\sigma_{10}$, $\sigma_{11}$ versus $1/\sigma_{11}$. The squares are values for the 0.01 g per dl solution; $\sigma_0$ versus $1/\sigma_0$, $\sigma_1$ versus $1/\sigma_1$, $\sigma_2$ versus $1/\sigma_2$, $\sigma_3$ versus $1/\sigma_3$, $\sigma_4$ versus $1/\sigma_4$, $\sigma_5$ versus $1/\sigma_5$, $\sigma_6$ versus $1/\sigma_6$, $\sigma_7$ versus $1/\sigma_7$, $\sigma_8$ versus $1/\sigma_8$, $\sigma_9$ versus $1/\sigma_9$, $\sigma_{10}$ versus $1/\sigma_{10}$, $\sigma_{11}$ versus $1/\sigma_{11}$, $\sigma_{12}$ versus $1/\sigma_{12}$, $\sigma_{13}$ versus $1/\sigma_{13}$, $\sigma_{14}$ versus $1/\sigma_{14}$, $\sigma_{15}$ versus $1/\sigma_{15}$. Several theoretical monomer-n-mer lines are indicated in the figure, based on a dimer molecular weight of 100,000 ($\sigma = 4$). The curve represents the predicted values for an indefinite associating system with a "monomer" molecular weight of 100,000.

**Fig. 6.** Estimation of minimum molecular weight of argininosuccinase. A (left), the relation of electrophoretic mobility to molecular weight of the SDS-polypeptide complexes of several standard proteins. B (right), the SDS-polypeptide complex of argininosuccinase from the same experiment. The gels for A and B were 7.5% polyacrylamide in 0.1 M sodium phosphate (pH 7)-0.1% SDS; 10 μg of protein were applied. The faint band near the end of the gel is the tracking dye.
A reduced, carboxymethylated preparation of the enzyme in 6 M guanidine hydrochloride, 0.1 M KCl, 0.02 M Tris chloride, 2 mM EDTA, pH 8.0, was centrifuged for 32 hours at 36,000 rpm at 20° in a 12-mm, three-channel centerpiece with a 2.4-mm liquid column. The absorbance base-line at the meniscus was ascertained after overspeeding to 44,000 rpm for 6 hours. The values have been corrected for the amount of bound carboxymethyl groups.

<table>
<thead>
<tr>
<th>Initial concentration</th>
<th>From linear least squares slope</th>
<th>$M_w$</th>
<th>$M_z$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.030</td>
<td>50,300 ± 1,000</td>
<td>51,200 ± 2,100*</td>
<td>51,200 ± 2,100*</td>
</tr>
<tr>
<td>0.015</td>
<td>51,200 ± 1,250</td>
<td>51,400 ± 2,100</td>
<td>52,200 ± 2,100</td>
</tr>
</tbody>
</table>

* The weight-average and z-average molecular weights of the entire sample were obtained by extrapolation of the point-average molecular weights to the base of the cell.

The absorbance of dinitrophenyl amino acids was determined after hydrolysis with 6 M guanidine hydrochloride at concentrations of 0.015 and 0.030 M, centrifuged in a three-channel centerpiece with a solvent-solvent pair, and a strictly linear relationship was also observed between ln recorder deflection versus $c$ in a straight line which coincided with the linear least squares plot. A molecular weight of 49,610 ± 1,100 was calculated from the slope.

Samples of a fully reduced and carboxymethylated preparation in 6 M guanidine hydrochloride at concentrations of 0.015 and 0.030 M were centrifuged in a three-channel centerpiece with a solvent-solvent pair, and a strictly linear relationship was also observed between ln recorder deflection versus $c$ in a straight line which coincided with the linear least squares plot. The molecular weights calculated from the linear least squares slopes and corrected for the presence of bound carboxymethyl groups are presented in Table I. The weight-average molecular weight and the z-average molecular weight of the entire solution were the same and were not affected by concentration. Similar molecular weight values were obtained for the protein in 2 M urea.

The absence of disulfide bonds is established since the molecular weight of the polypeptide chains in guanidine hydrochloride or SDS was not influenced by reducing agents and the total half-cystine content determined after performic acid oxidation of the protein was equal to the number of free thiol groups (cf. "Number of Sulphydryl Groups"). The foregoing evidence indicates that these four polypeptide chains correspond to the minimum subunit or monomer, and that the native enzyme, of molecular weight 202,000, is a tetramer composed of four subunit polypeptide chains of identical molecular weight, 50,400 ± 1,100. This is the average value of the experimental data discussed above.

**Identity of Subunits**

**NH₂-terminal Amino Acid**—All attempts to identify the NH₂-terminal residue were unsuccessful. Small amounts of the phenylthiohydantoin derivatives of glycine, serine, and alanine were detected by thin layer chromatography after one cycle of the Edman degradation, as modified by Lai. Amino acid analysis of the phenylthiohydantoin derivatives after alkaline hydrolysis showed only 0.4 mole of glycine, 0.2 mole of alanine, and 0.2 mole of serine per 200,000 g of protein; these concentrations are too low to be considered significant. Dinitrophenylation was carried out by treating the subunits in 2 M guanidine hydrochloride, 0.05 M NaHCO₃ at 40° with 1-fluoro-2,4-dinitrobenzene. No α-dinitrophenyl amino acid was detected either in other extracts or water extracts (32) of acid hydrolysates of the dinitrophenylated protein. Amino terminal determinations were also made according to the cyanate method of Stark and Smyth (33). Glycine, alanine, serine, and aspartic acid were found in quantities between 1 and 2 moles per mole of protein both in the cyanate-treated protein sample and in the protein blank carried through the procedure in the absence of cyanate. After subtraction of the blank values, the concentrations of these amino acids were 0.4 mole of alanine, 0.1 mole of glycine, and 0.1 mole of serine per mole of protein. These results suggest the amino-terminal residue may either be one of these amino acids, or alternatively, the end groups may be blocked, perhaps by acetylation.

**COOH-terminal Amino Acid**—Hydrazinolysis of carboxymethylargininosuccinase at 110° for 6 and 14 hours in four experiments released, per mole of enzyme, only small amounts of glycine (0.7 to 1.0 eq), serine (0.2 to 1.0 eq), and alanine (0.2 to 0.5 eq).

Preliminary experiments showed that the native enzyme was not hydrolyzed by DFP-carboxypeptidases A and B; therefore, the digestions were carried out on denatured protein in 2 M urea or 10⁻³ M SDS or maleylated protein. High enzyme to substrate ratios were required for hydrolysis, and the addition of carboxypeptidase B did not alter the pattern of amino acids released.

Table II is a summary of the results of amino acid analyses after digestion of argininosuccinase with DFP-carboxypeptidase A. The amide constituent of the "serine + asparagine + glutamine" peak was determined in Experiment 2 by hydrolyzing a portion of the 14-hour sample in 6 M HCl at 110° for 22 hours and analyzing for the increase in glutamic acid and aspartic acid. After correction for the carboxypeptidase A blank, only an increase in glutamic acid, equivalent to the decrease in the composite peak, was observed. This indicates that glutamine and not asparagine was released from the COOH-terminal sequence. In Experiment 1 and in the 4-hour run of Experiment 2, the analyses show that the peak corresponding to "serine + glutamine" was released in the greatest amount, suggesting that glutamine is the carboxyl-terminal amino acid. Although 4 residues of glutamine were not found, the difference may be explained by the amount of glutamic acid. The fact that after acid hydrolysis a total of 4 eq of glutamic acid were obtained suggests that glutamine and glutamic acid may both be carboxyl-terminal. Glutamic acid is known to be released by carboxypeptidase A more slowly than glutamine.

Although the data in Experiment 2 show that these amino acids are present in equal amounts, this ratio may be fortuitous. Based on the assumption that the glutamic acid end groups arose during the isolation procedure as a result of partial amide hydrolysis of glutamine end groups, some variation should be expected from one preparation to another.

Glutamine was also identified as its DNS-derivative. A DFP-carboxypeptidase A hydrolysate of maleylargininosuccinate (prepared as for Experiment 3) was treated with DNS-chloride, and the DNS-glutamine acids were separated as described under "Experimental Procedure." Given in order of fluorescent intensity, the following DNS-amino acids were found: glutamine ≥ leucine ≥ alanine > glycine ≥ valine > serine ≥ lysine > glutamic acid. The material in the spot corresponding to DNS-glutamine was recovered by two extractions with ethyl acetate. The solvent was removed and the DNS-glutamine was hy-
drolyzed with 6 N HCl for 50 min at 120° in an autoclave. After removal of the HCl, the material was rechromatographed. The most intense spot now corresponded to DNS-glutamic acid; there was also a second very much smaller spot corresponding to DNS-glutamine.

Because neither leucine nor alanine were found in significant amounts after hydrazinolysis, glutamine must be the carboxyl-terminal amino acid. This explains the failure to identify the COOH-terminal residue by hydrazinolysis, as the monohydrazide of glutamic acid is not recovered with the procedures used here to separate the bulk of the hydrazides from the free amino acids (35), and glutamic acid is recovered in low yield after hydrazinolysis. The overlap of glutamine with serine on the automatic amino acid analyzer prevents a direct quantitation of glutamine. In Experiment 3 where conditions favored complete hydrolysis of several residues in the terminal sequence, the release of 4.02 eq of leucine, the penultimate amino acid, and of 3.88 eq of alanine is strong evidence for the presence of 4 moles of COOH-terminal amino acid per mole of protein. Based on the rate of release, the tentative carboxyl sequence of the enzyme is \((\text{Val, Ser, Gly})\)-Ala-Leu-Gln-COOH (and -Leu-Glu-COOH).

**Cleavage with Cyanogen Bromide**—In proteins composed of multiple polypeptide chains, differences in amino acid composition or sequence can be estimated from the number of fragments obtained after the methonyl peptide bonds are cleaved with cyanogen bromide. As determined by amino acid analysis (4) 1 mole of argininosuccinase contains 51 residues of methionine, corresponding to an average distribution of 13 residues per chain. If the polypeptide chains are identical, quantitative cleavage at methionine should produce 14 peptides.

A carboxymethylated preparation of argininosuccinase was treated with cyanogen bromide and the reaction products were separated as described under "Experimental Procedure." Amino acid analyses of the carboxymethylated protein before and after cyanogen bromide treatment showed that: the destruction of methionine was complete; the sum of homoserine and homoserine lactone was nearly equimolar with the original methionine content (91%), and the number of all of the other amino acid residues remained unchanged.

The cyanogen bromide cleavage products were separated by polyacrylamide gel electrophoresis at pH 8.9, and 14 bands of varying intensity were observed after staining with Coomassie blue. Four of these bands were very dark, whereas three stained very lightly and their presence was substantiated by scanning in the Gillford spectrophotometer. There was no material at the origin. Of the 10 bands that were observed after polyacrylamide gel electrophoresis at pH 2.7, six were heavily stained. This difference in the number of bands found with the two electrophoretic systems suggests inadequate separation at pH 2.7, possibly caused by the presence of Triton. The cyanogen bromide peptides were also separated on paper by high voltage electrophoresis at pH 3.5, and 13 peptides, of which one migrated as an anion, were visualized with ninhydrin. These results are in excellent agreement with the 14 fragments predicted on the assumption that argininosuccinase is composed of four identical polypeptide chains.

**Trypsic Peptide Maps**—Preparations of carboxymethylargininosuccinase (with and without 14C-labeling) were digested with TPCK-trypsin as described under "Experimental Procedure." Approximately 2 mg of material were used to obtain each peptide separation. The peptide patterns were developed sequentially for specific amino acids as described by Easley (38) and Smith (37); the ninhydrin reaction was used to determine the total number of peptides. A summary of the data obtained by this technique is presented in Table III. Peptides containing

<table>
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<th>Table II</th>
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<tr>
<td><strong>Hydrolysis of argininosuccinase with carboxypeptidase A</strong></td>
</tr>
</tbody>
</table>
| The protein substrate (0.5 to 1.2 mg per ml) was digested in 0.2 M N-ethylmorpholine, pH 8.5, under the conditions indicated in the table. The amount of carboxypeptidase A in each experiment is given in relation to substrate expressed as a w/w ratio. Substrate abbreviation is M- for maleyl-.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Amino acids released</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(Serine and glutamine)</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>Cm-Aargininosuccinase, 2 M urea, 25°C; 1/30</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>Argininosuccinase, 0.1 M SDS, 37°C; 1/25</td>
</tr>
<tr>
<td>1 hrs.</td>
<td>(4.28)</td>
</tr>
<tr>
<td>8 hrs.</td>
<td>(4.50)</td>
</tr>
<tr>
<td>14 hrs.</td>
<td>2.45</td>
</tr>
<tr>
<td>14 hrs.</td>
<td>2.45</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>M-Aargininosuccinase, 30°C; 1/10</td>
</tr>
</tbody>
</table>

* Because of the poor resolution in this region, the sum of these amino acids is an estimate, and therefore is given in parentheses.
* After hydrolysis in 6 N HCl at 110° for 22 hours.

<table>
<thead>
<tr>
<th>Table III</th>
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<tbody>
<tr>
<td><strong>Summary of analysis of tryptic peptide maps</strong></td>
</tr>
<tr>
<td>The data were obtained from three peptide patterns of carboxymethylargininosuccinase. The samples were prepared as described under &quot;Experimental Procedure.&quot;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Residue specificity</th>
<th>Number of peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found</td>
<td>Predicted*</td>
</tr>
<tr>
<td>Ninhydrin</td>
<td>Total number of peptides</td>
<td>48-51</td>
</tr>
<tr>
<td>Diazonium salt of sulfinic acid, (Pauly)</td>
<td>Histidine and tyrosine</td>
<td>21</td>
</tr>
<tr>
<td>α-Nitrosyl-β-naphthol</td>
<td>Tyrosine</td>
<td>9</td>
</tr>
<tr>
<td>Platinic iodide</td>
<td>Methionine and Cm-cysteine</td>
<td>17</td>
</tr>
<tr>
<td>Radioautography</td>
<td>[14C]Cm-peptides</td>
<td>4</td>
</tr>
</tbody>
</table>

* Maximum number per mole of 202,000, based on quantitative amino acid analyses (4).
histidine residues were identified on the basis of a positive reaction with the Pauly reagent and the absence of a reaction with α-nitrosoy-β-naphthol. Methionyl and carboxymethyllys-teinyl peptides were differentiated by the rate of reaction with platinic iodide. Radioautographs, used to locate the carboxymethyllysine peptides, showed four major radioactive peptides but also showed several less radioactive spots which corresponded to peptides containing histidine, and one spot that apparently contained methionine.

The data in Table III show that 48 to 51 ninhydrin-reactive peptides were found after tryptic digestion. Since argininosuccinase contains 76 tryptic residues and 116 arginyl residues (4), only one-fourth of the total possible number of peptides were observed. One-fourth of the maximum number of predicted peptides predicted from amino acid analysis were found to contain histidyl, tyrosyl, cysteinyl, and methionyl residues.

Argininosuccinase consists of four subunit polypeptide chains of equal molecular weight; cyanogen bromide cleavage and tryptic digestion gave one-fourth of the maximum number of products, and COOH terminus studies suggest four chains of similar carboxyl-terminal sequence. On the basis of these criteria the subunits are very similar, if not identical, in their amino acid sequence.

### Relation of Sulfhydryl Groups in Argininosuccinase to Tetrameric Structure

#### Number of Sulfhydryl Groups—When the number of sulfhydryl groups in the native enzyme was determined at 25°C in Tris or phosphate buffer at pH 7 with 2 mM DTNB as described under “Experimental Procedure,” 3.8 to 4 moles of —SH per mole of enzyme were found. Under these conditions no further increase in absorbance was observed even after several hours. In the presence of denaturing agents, the number of reactive —SH groups per mole of enzyme increased to a total of 14 to 16. This value agrees with the number of half-cystine residues found by amino acid analysis. After carboxymethylation, 15.9 moles of carboxymethyllysine were found per 202,000 g of protein, and after performic acid oxidation, 16 moles of cysteic acid. The data are summarized in Table IV. Taken together, the results indicate that the 16 half-cystine residues are all in the sulphydryl form. Four are readily available to sulphydryl reagents; the other 12 are presumably buried in hydrophobic regions and are inaccessible except under dissociating conditions.

#### Effect of Sulfhydryl Reagents on Enzyme Activity—Earlier work had shown that sulphydryl reagents do not inhibit the catalytic activity of argininosuccinase. However, it was observed that the addition of p-HMB or Mercapto to cold-inactivated enzyme completely prevented reactivation; iodoacetic acid and iodoacetamide had no effect, and N-ethylmaleimide had only a partial effect (1). Those observations were confirmed and extended in the present study. Catalytic activity in both Tris and phosphate buffers was not affected by 1 mM p-HMB or 5 mM iodoacetate when measured directly in the assay cuvette in the presence of substrate, even when the enzyme was exposed to the reagent in a prior incubation in the absence of substrate. In separate experiments where it had been ascertained that 4 moles of —SH per mole of enzyme had reacted with DTNB or p-HMB, aliquots taken from such reaction mixtures and assayed directly for catalytic activity were found to retain their full activity. The four thiol groups that react readily with sulphydryl reagents are evidently far removed from the active site, since blocking four —SH groups, as well as the introduction of bulky residues, has no effect on the catalytic properties of the enzyme.

#### Effect of Sulfhydryl Reagents on Reversible Cold Dissociation—

The same reagents have no effect on the rate or the extent of dissociation in the cold, as measured by the loss of activity. When the rate of dissociation was followed during incubation at 0°C in a mixture containing 0.1 M Tris chloride, pH 9.0, 2 mM EDTA, and 1 × 10^{-4} or 4 × 10^{-4} M p-HMB (8 or 32 moles of p-HMB per mole of protein) or 0.01 M iodoacetate, the first order rate constants were the same as the control without sulphydryl reagent. At the end of each experiment samples were reactivated by incubation at 38°C in 0.1 M potassium phosphate, pH 7.5. The control and the sample containing iodoacetate regained 100% of the original activity, but the sample containing p-HMB did not regain any activity and precipitated out of the solution during incubation at 38°C. The effect of p-HMB in preventing reactivation could be reversed by adding an excess of cysteine or glutathione just prior to incubation at 38°C. Although almost all of the original activity (91%) was regained, the enzyme, reactivated this way, seemed to be less stable since incubation at 38°C for a longer period resulted in a subsequent slow loss of activity with time.

As the rate of cold dissociation is unaffected by p-HMB, it seems that p-HMB does not itself promote dissociation, but that the process of dissociation to dimers exposes a further number of reactive sulphydryl groups. The binding of p-HMB to these sulphydryl groups subsequently interferes in some way with reactivation.

If this hypothesis is correct, the number of available sulphydryl groups should increase during cold dissociation. Argininosuccinase was subjected to cold dissociation; after 70% inactivation, analysis of small aliquots showed that 6.9 moles of —SH per mole of protein could react with DTNB. The 70%-inactivated enzyme was incubated at 38°C and the original specific activity was restored. Sulphydryl estimation (with DTNB) now showed that 4.1 moles of reactive sulphydryl were available per mole of protein. These results support the concept that dissociation to dimers, even in the absence of denaturing agents, uncovers a further number of previously unreactive sulphydryl groups. After the native tetramer has been restored by reassociation,
The rate constants of these two reactions, reassociation and the reaction of $-$SH groups with DTNB, are of the same order of magnitude.

The reaction of DTNB with the 16 sulphydryl groups was 98% complete in 1 min when measured after dissociation in SDS.

### Correlation of Cold Dissociation with Release of Newly Accessible Sulphydryl Groups

In the following experiments the rate of reaction of $-$SH groups with DTNB was not a rate-limiting factor with respect to the rate of cold dissociation. The reaction with DTNB was measured at 25°C; it was 95% complete in 2 min with the native enzyme, about 90% complete in 2 min with the dimer, and 98% complete in 1 min in the presence of guanidine hydrochloride.

A solution of argininosuccinase was dissociated by incubation at 0°C in 0.1 M Tris chloride, pH 8.6, containing 2 mM EDTA, and the change in catalytic activity and the number of sulphydryl groups were measured as a function of time. Because some reassociation of the enzyme was expected at 25°C during the 30-min time period required for completion of the reaction with DTNB, enzymatic activity was determined at the end of the 30-min period with aliquots taken from the DTNB-containing reaction mixture. Thus, in the following experiments the rate curves are displaced 30 min on the abscissa, although the slope is unchanged. When the number of $-$SH groups was plotted against the per cent of initial activity (percentage of undisso-

### Stepwise Release of $-$SH Groups

The stepwise manner of dissociation of the enzyme could be discerned by $-$SH titration under dissociating conditions in the presence of the titrating reagent, DTNB. With the native enzyme in 0.1 M potassium phosphate, pH 7.5, only 3.8 to 4.2 $-$SH groups are measurable in the presence of DTNB even after 48 hours at 2°C. In Tris or imidazole buffer the $-$SH groups reacted with DTNB more slowly at 2°C (than at 25°C), and after 30 min four $-$SH had re-

### Example

When 2 moles of DTNB (over the zero time value) have reacted per mole of protein, 50% of the enzyme was undisso-

### Conclusion

This all-or-none release of four newly accessible $-$SH groups was independent of protein concentration (as was dissociation), and, thus, allowed a comparison of the rate of cold inactivation (dissociation) with the rate of release of sulphydryl groups. The rate of cold dissociation was plotted according to the first order rate equation $log a/a = -kt/2.3$, where $a$ is 100% inactivation and $x$ is percentage of inactivation at time $t$. The rate of appearance of sulphydryl groups was calculated from

$$log \frac{[SH]_{100\%\, diss}}{[SH]_{100\%\, diss} - [SH]_0} = kt/2.3$$

where $[SH]$ represents moles of $-$SH per mole of protein.
At \( k_{-2.3} \), where \( A_f \) is the final absorbance at the end of the reaction, Fig. 11 is a near-focus micrograph of argininosuccinase. The images were recorded photographically on Ilford EM-6 plates in a through-focus series. The figure presented here, Fig. 11, is a near-focus micrograph of argininosuccinase. It is the middle of three photographs in the parafocal region with a potential resolution limited to 5 to 7 Å by the phase image granularity.

In all of the preparations examined, and as scrutiny of Fig. 11 shows, the molecules appeared both as triangles and as squares with an indication of four subunits. Images of dimers and crosses appeared less frequently. In the best defined structures the subunits appeared to be approximately spherical. In other micrographs taken at moderate underfocus, the individual subunits in both the triangular and square forms were unambiguously identifiable.

The images shown in Fig. 11, A through E, magnified 1,000,000 times, illustrate the several views presented by the most favorably stained molecules. Two triangular orientations are represented in A and B, whereas C shows a tetrad in rectangular orientation. In D the image appears as a sphere with little stain penetration between subunits. The dimeric form in E, with outer dimensions of 95 to 100 × 52 Å, clearly shows the outline of the individual subunits. As may be seen in the main field, × 260,000, and in the middle photograph at higher magnification, × 320,000, the most common molecular views can be described as being approximately triangular and rectangular. Such views strongly suggest C4 symmetry rather than C3 symmetry. A tetrahedral model provides an explanation for the orientation of the majority of well contrasted molecules observed in a field. It also suggests that the subunits are arranged very compactly, and that stain penetration into the intersubunit bonding areas is limited. Molecules that show no apparent subunit structure yet in outer contour appear square or triangular have also been observed.

Using the most enlarged images, an estimate of subunit diameter was made from measurements of molecules that were obvious dimers. The average center-to-center distance was 45 Å. The molecular weight of the subunit is 50,400, and with a specific volume of 0.734 this would correspond to a diameter of 49 Å, assuming the subunit to be a solid sphere. The images in Fig. 11 are sufficiently clear to consider these results as supporting evidence of the four-subunit model derived from chemical and physical data.

**DISCUSSION**

Under selected conditions argininosuccinase dissociates preferentially to a dimer. Clearly, at least two kinds of subunit interactions can be distinguished in this dissociation. The bonds holding two dimers together (tetramer bonds) are the weaker interactions, and these bonds are characterized by their susceptibility to dissociation at low temperature and low ionic strength. The bonds that hold monomers together (dimer bonds) are ruptured by high concentrations of guanidine hydrochloride and urea, and by SDS.

In recent reviews on the quaternary structure of enzymes (38, 39), it has been suggested that molecular symmetry can be predicted from studies of dissociation behavior. If the dissociation product or products of a system can be defined and if a preferred mode of dissociation occurs, certain assumptions can be made by definition of the rules governing symmetry. The intersubunit binding sets of subunits with cyclic (C4) symmetry are of only one kind, and, hence, all of these bonds must be identical in terms of strength. Dihedral (D2) symmetry, on the other hand, requires at least two different types of binding sets. On the basis of the two different binding sets described here for argininosuccinase, a model with D2 symmetry is highly favored. The tetrahedral arrangement suggested by the electron microscope studies also implies D2 symmetry; moreover, it implies three binding sets per protomer.

One binding set, the strongest of the three, is the interaction...
between two monomers to form an isologous dimer. The other two postulated binding sets between the dimers may be indistinguishable. Hydrophobic bonds have been implicated in the dimer interactions from the negative temperature dependence, the small negative free energy, and the large positive entropy values calculated from the association constant in phosphate at pH 7.5 (2). The protection by phosphate and by substrates is effected by strengthening the bonds at these sites. The changes induced by binding these ligands result therefore in changes in quaternary conformation.

Acknowledgments—We are deeply indebted to Dr. C. Y. Lai for his invaluable guidance during the end group studies, and to Dr. Lai and Dr. B. L. Horecker for so generously extending their laboratory facilities to us for these studies and for the many amino acid analyses.

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