Subunit Structure of Aspartate Transcarbamylase from Escherichia coli*

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(Received for publication, September 10, 1970)

SUMMARY

1. The molecular weights of the catalytic and regulatory polypeptide chains of aspartate transcarbamylase were determined to be 33,500 and 17,000, respectively. The results of three empirical methods are in good agreement with those obtained by sedimentation equilibrium in guanidine-hydrochloride or derived from the intrinsic viscosity and the sedimentation coefficient measured in the same solvent. For the regulatory chain, all values obtained are in the range of 17,000, in agreement with the amino acid sequence (WEBER, K., Nature, 218, 1116 (1968)).

2. The molecular weights of the native enzyme and the catalytic subunit were redetermined. The two proteins have masses of 300,000 and 100,000 daltons and show no association-dissociation equilibrium. From the molecular weights of the catalytic subunit and its polypeptide chain, we conclude that this subunit is a trimer.

3. Three independent techniques indicate that the catalytic and the regulatory polypeptide chains are present in equal numbers. This result, in combination with the molecular weights of these chains, indicates that the enzyme molecule contains six catalytic and six regulatory polypeptide chains which are held together solely by noncovalent bonds.

4. Renaturation of aspartate transcarbamylase and the catalytic subunit was achieved from guanidine-hydrochloride or dodecyl sulfate solutions. The reactivated native enzyme and catalytic subunit are indistinguishable from their untreated counterparts. A subunit identical in every respect with catalytic subunit isolated after p-hydroxymercuribenzoic acid. For the purified one, 45,000 to 50,000 d, a value again considerably below the expected one of 60,000 to 80,000 (5). On the basis of these results and the mass distribution between the two subunits in the native enzyme (8), Weber proposed a hexameric structure for aspartate transcarbamylase. The tetrameric model, suggested previously, was incompatible with the molecular weights either of the polypeptide chains of the native enzyme and its subunits. The crystallographic data obtained at that time by Wiley and Lipscomb (9) also ruled out a tetrameric arrangement, but were in agreement with a hexameric configuration.

5. The determination of the number of binding sites for the substrate analogue succinate in the catalytic subunit yields a value in agreement with the proposed model. Further support for a hexameric structure comes from the fact that aspartate transcarbamylase contains six tightly bound zinc ions. The metal ions are not required for catalysis and can be replaced by cadmium in vivo or in vitro.

Since Gerhart and Schachman found that the catalytic and the regulatory functions of aspartate transcarbamylase from Escherichia coli are located on different and separable subunits1 (2), this enzyme has attracted wide interest and has served as a model system for the testing of theories concerning allosteric mechanisms (9). For some time, its structure was thought to be that of a tetramer containing four catalytic and four regulatory polypeptide chains (4). This conclusion was based on the determination of the molecular weights of the native enzyme (310,000) and its subunits (2), the number of binding sites for substrates and effectors (4), and on determinations of the molecular weights of the polypeptide chains by a number of physical (5) and chemical techniques (5, 6).

When Weber (7) established the amino acid sequence of the regulatory protein, it became clear that the molecular weight of this polypeptide chain was 17,000 rather than 27,000, the value anticipated from previous data. Confirmation of this result was obtained by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and by carboxyl end group analysis (7). These latter two techniques yielded a molecular weight for the C chain of approximately 33,000, a value again considerably below the expected one of 45,000 to 50,000 (5). On the basis of these results and the mass distribution between the two subunits in the native enzyme (8), Weber proposed a hexameric structure for aspartate transcarbamylase. The tetrameric model, suggested previously, was incompatible with the molecular weights either of the polypeptide chains of the native enzyme and its subunits. The crystallographic data obtained at that time by Wiley and Lipscomb (9) also ruled out a tetrameric arrangement, but were in agreement with a hexameric configuration.

1 The nomenclature for oligomeric proteins was adopted from Monod, Wyman, and Changeux (1), with the exception of the term "subunit," which these authors left purposely undefined. In connection with aspartate transcarbamylase, it is operationally defined to mean the submolecular entities (comprised of identical polypeptide chains) which are obtained by dissociation of the native enzyme by p-hydroxymercuribenzoic acid. For the purpose of a clearer presentation, we suggest the symbols C and R for "catalytic" and "regulatory" when referring to the particular subunits or polypeptide chains.
Such a structure, however, would imply that the enzyme has some unusual features. If the molecular weight of the catalytic subunit is 100,000, as estimated by Gerhart and Schachman (2), and that of the C polypeptide chain is 33,000, the stable and enzymatically active C subunit would be a trimer. Odd numbered oligomers, however, are thought to be dis favored by selection (1, 10). Also surprising is the discrepancy between four binding sites and six catalytic polypeptide chains, although such a difference is possible in principle (11).

These problems led us to reinvestigate the characteristics of the enzyme which are relevant to its subunit structure. We have redetermined the molecular weights of the polypeptide chains, the native enzyme, and the subunits. The number of binding sites for succinate, as well as for metal ions, was examined. Furthermore, we sought evidence for the assumption that the catalytic subunit, as obtained by HMB dissociation, is indeed a structure which is preserved in the native enzyme. Our results are in agreement with a hexameric configuration for this molecule and allow us to propose a geometrical arrangement of the polypeptide chains in this enzyme.

**EXPERIMENTAL PROCEDURES**

**Enzyme**—Aspartate transcarbamylase was prepared from a uracil-requiring strain kindly provided by Dr. J. Gerhart. The enzyme was purified and dissociated into its subunits by HMB as described by Gerhart and Holoubek (12). The only modification of the original procedure was the use of potassium phosphate buffer (4 mM, pH 7.0) rather than imidazole-HMB buffer in the stepwise elution of the subunits from DEAE-Sephadex. The separated subunits were freed from the mercurial by dialysis against 3.6 mM ammonium sulfate containing 0.1 mM \( \beta \)-mercaptoethanol and 0.1 mM EDTA. The proteins were stored as ammonium sulfate precipitate at 4°C.

Catalytic subunit can also be prepared by heat cleavage. A dilute enzyme solution (2 mg per ml) in 4 mM potassium phosphate, pH 7.0, containing 0.4 mM EDTA and 2 mM \( \beta \)-mercaptoethanol, was heated for 8 min at 64°C in a waterbath, then rapidly cooled in ice and the precipitate centrifuged out. The yield of C subunit in the supernatant was 50 to 70% with respect to the amount initially present. If higher enzyme concentrations were used, yields were lower.

Enzyme concentrations were determined spectrophotometrically using the absorption coefficient \( \epsilon_{280,0.1cm} = 0.59 \) for aspartate transcarbamylase and 0.70 for the C subunit. The R subunit was quantitated by amino acid analysis or by the method of Lowry et al. (13) Enzymatic activity was assayed by a modification of the continuous assay system (14) in a pH-stat (Radiometer, Copenhagen). The pH of the reaction mixture was kept at 8.3.

**Chemicals**—Urea and sodium dodecyl sulfate were recrystallized from ethanol. Guanidine hydrochloride was recrystallized (15), or purchased in its “ultra pure” form from Mann Research Laboratories. Sucrose “ultra pure” and phenylmethane sulfonylfluoride were from the same company. \( p \)-Hydroxymercureni benzoate was obtained from Sigma, and 5,5’dithiobis(2-nitrobenzoic acid) (A grade) was purchased from Calbiochem. Radiochemicals were from New England Nuclear Corporation with the following specific activities: 10 Ci per mole for succinic-2,3,4,5C

1 The abbreviation used is: HMB, \( p \)-hydroxymercureni benzoi acid.

2 F. Quiocho, to be published.
ducted in an analytical centrifuge equipped with a photoelectric scanning absorption system (22). Absorption was recorded at 230 μm. The buffer system used was identical with the one described above except that the solution was 1% in sucrose and contained no β-mercaptoethanol. We did not observe instability of the catalytic subunit under these conditions for the length of a run. Protein concentrations varied between 30 to 150 μg per ml.

Sedimentation Velocity Experiments—These were performed using Schlieren optics. For the determination of sedimentation coefficients in buffer, single sector cells were used at rotor speeds of 50,740 rpm. Values of s in 6 mM guanidine hydrochloride were determined in a synthetic boundary cell of the capillary type at 24,040 rpm. All sedimentation coefficients were corrected to the density and viscosity of water at 20° (23, 24), if not indicated otherwise.

Zone Centrifugation in Sucrose Gradients—Protein solutions (0.05 to 0.3 ml) were layered onto linear gradients (4.5 ml) containing 5 to 20% sucrose in 40 mM phosphate buffer, pH 7.0, and centrifuged at 64,000 rpm in a titanium rotor equipped with three swinging buckets in a Spinco model L-2 ultracentrifuge. Centrifugation was performed at 4° for 6 hours with aspartate transcarbamylase and for 12 hours with subunits. Positions of proteins were determined by monitoring optical density and enzymatic activity of the fractions. When the purpose of the experiment was the determination of sedimentation coefficients, well characterized proteins were used as standards. The s value of test proteins was obtained by the formula of Martin and Ames (25), and their approximate molecular weights by the equation derived by Mandelkern and Flory (26).

Viscometry in Guanidine Hydrochloride—Measurements were made using Ostwald-Cannon-Manning semimicro type viscometers. Flow times of the five instruments used were in excess of 220 sec for water. They were measured to 0.001 min and the reproducibility obtained was of the order of 1 part in 1000. The temperature of the waterbath was regulated to 25° ± 0.005°. Solutions of guanidine hydrochloride (6 M, and 0.1 M in β-mercaptoethanol) were tested and the values found for viscosity and density were in excellent agreement with those given by Kawahara and Tanford (24). Protein concentrations were obtained by two methods. Proteins were lyophilized from 1 mM am-

Solutions of guanidine hydrochloride (6 M, and 0.1 M in β-mercaptoethanol), and 50 mM Tris-HCl, pH 7.5. This buffer was used to insure rapid reduction of the protein. Alternatively, stock protein solutions containing approximately 40 mg per ml of protein in 0.2 M Tris-HCl, pH 7.5, were prepared and their exact protein content was determined from their optical density and by amino acid analysis. One part of the solution was then dissolved in three parts of solvent containing 6 M guanidine hydrochloride and β-mercaptoethanol to give final concentrations of 6 M and 0.1 M, respectively. Serial dilutions were made using volumetric pipettes. Reference solvents were treated identically and 1-ml portions were applied to the viscometer. The intrinsic viscosity of bovine serum albumin was measured to judge the experimental accuracy. As seen in Fig. 2, a value of 52.2 ce per g was obtained, in agreement with the value of 53.0 ce per g, given by Tanford, Kawahara, and Lapanje (27).

Gel Filtration Experiments—For the determination of molecular weights of aspartate transcarbamylase and its subunits in buffer, samples were chromatographed at 4° on Sephadex G-200 in a column equipped with flow adapters for ascending chromatography. The bed volume was 360 ml and a phosphate buffer (40 mM sodium phosphate, 0.1 M NaCl, pH 7.2) was used. The buffer flow was 12 to 14 ml per hour, and fractions of 3.5 ml were collected. Samples (1% of the total bed volume) were 0.2% in blue dextran and contained either 5 mg of sucrose or 0.1 μCi of 14C-alanine to indicate the exclusion volume (V0), and the volume of solvent contained within and without the gel matrix (V0), respectively. Elution volumes (V0) of proteins were determined by monitoring at 230 μm. Catalase and myoglobin were also followed at 410 μm. β-Galactosidase (28), aspartate transcarbamylase, and catalytic subunit were assayed by their enzymatic activity. Blue dextran was monitored at 620 μm and sucrose was assayed with anthrone reagent (29). Radioactivity was measured by dissolving 50 μl of the effluent in 10 ml of Kinard's solution (30) and counting in a liquid scintillation counter. Distribution coefficients (Kd) of proteins were calculated according to Andrews (31).

The column was calibrated with eight typical globular proteins. Two to four of these standards were applied together in amounts of 0.2 to 10 mg per ml each. β-Galactosidase was added routinely in order to check column performance. All of the eight proteins used have frictional ratios between 1.1 and 1.35 and partial specific volumes of 0.73 to 0.76 ce per g (32). None of them exhibits noticeable association or dissociation at the concentrations and under the conditions used. When their respective Kd values were plotted versus the logarithms of their molecular weights, a straight line was obtained (Fig. 5). Similarly, plots of Kd values versus the logarithms of either sedimentation or diffusion coefficients yielded straight lines (32). Stokes radii, evaluated from the experimental Kd values and the pore radius of Sephadex G 200 according to the procedure of Acker (34), agreed well with those calculated (33) from the known diffusion coefficients of the standards. This indicated the suitability of the proteins chosen as standards (34, 36-38). The molecular weights of the test proteins were estimated from the distribution coefficient and the calibration curve shown in Fig. 5. Since this method is only valid if the proteins are globular, their frictional ratios were calculated from the Stokes radius in combination with the molecular weight obtained by sedimentation equilibrium (39).

Gel filtration in 6 M guanidine hydrochloride ("ultra pure," Mann) was performed as suggested by Davison (40), using the procedure of Fish, Mann, and Tanford (41). Bio-Gel A, 5 m (200 to 400 mesh) with an agarose content of 6% was used. The column dimensions were 0.9 × 80 cm. The exclusion volume was marked by blue dextran (0.3% w/v) and V0 was indicated by the addition of dinitrophenylalanine (0.03%). All proteins used were reduced and carboxymethylated according to standard techniques (42). This procedure avoided the addition of β-mercaptoethanol to the column buffer, and proteins could be moni-

* Aldolase (10 mg) was used in these experiments, so that concentrations in the peak tubes were well above the critical value of 0.2 mg per ml, below which aldolase has been reported to dissociate (33).
tored by optical density measurements at 230 nm. Since the R chain has a low absorption, its elution volume was also determined by using aspartate transcarbamylase which was carboxymethylated with tritiated iodoacetic acid. The enzyme was carefully freed of β-mercaptoethanol by dialysis and brought to pH 10.5 by the addition of sodium bicarbonate buffer (1 M, pH 10.8) to a final concentration of 0.1 M. ∝ H Iodoacetoate was added and the reaction allowed to proceed for 15 min. The solution was then made 6 M in guanidine hydrochloride, 0.1 M in β-mercaptoethanol, and 0.1 M in Tris and brought to pH 8.3 by the addition of 1 N HCl. After 4 hours of incubation, the protein was allowed to react extensively with unlabeled iodoacetic acid. In this way, any desired fraction of the lysyl residues of the protein could be reacted by the addition of limiting amounts of ∝ H-iodoacetoate, and thus the number of radioactively labeled sites could be increased. Extensive reduction and carboxymethylation at the lower pH insured full dissociation of the protein. After dialysis into the column solvent (0.1 M Tris-acetate, pH 8.2, 5% of the published result.

Determination of cysteine residues was carried out using 5,5'-dithiobis(2-nitrobenzoic acid), essentially as described by Sedlak and Lindsay (46). Protein solutions were freed from β-mercaptoethanol by gel filtration through Sephadex G-25. Protein concentrations were determined spectrophotometrically or by amino acid analysis. Care was taken to exclude oxygen from the reaction mixtures. Aliquots were mixed with a solution of 8 M guanidine hydrochloride and 3 × 10⁻⁴ M in 5,5'-dithiobis(2-nitrobenzoic acid), using 0.2 M Tris-acetate, pH 9.2, as a buffer. The final guanidine hydrochloride concentration was 6 M. Absorbance at 412 nm was read immediately against a blank without protein. The extinction coefficient used was ε₁₄₈ = 1.36 × 10⁴, the value given by Eilman (47).

Amino acid analyses were performed by the method of Spackman, Stein, and Moore (48). Proteins were hydrolyzed under vacuum for 24 hours in 6 N HCl at 109°. Half-cystines were determined as cysteic acid after oxidation with performic acid (43).

**Determination of Binding Sites**—The number of binding sites for succinate in the C subunit and the dissociation constant of the complex were determined by the method of Colowick and Womack (49). Porter, Modebe, and Stark (50) have reported that the binding constant for succinate increases with decreasing pH and that sodium cacodylate has no inhibitory effect on enzymatic activity. We therefore chose this buffer (50 mM) at a pH of 6.6, at which succinate is practically entirely ionized. Since catalytic subunit has a tendency to precipitate in this buffer at 4°, enzyme solutions were subjected to high speed centrifugation prior to use. C subunit (6 to 9 mg per ml), carbamylphosphate (4.8 × 10⁻⁴ M), and ¹⁴C-succinate (3 × 10⁻⁶ M) were introduced into the upper chamber of the dialysis cell. The ligand concentration was increased stepwise to a concentration of 9 × 10⁻⁴ M by the addition of ¹⁴C-succinate. A final increase to 2 × 10⁻³ M allowed us to determine the free dissociation rate. Protein concentrations were measured spectrophotometrically, and the rate of dissociation was determined by counting radioactivity in the effluent of the lower compartment (capacity 1.7 ml). The flow rate of the buffer was 4.5 ml per min, so that the time required for an experiment was approximately 40 min.

The stability of carbamylphosphate was estimated in potassium phosphate buffer (40 mM, pH 7.0, containing 2 mM β-mercaptoethanol and 0.2 mM EDTA) using ¹⁴C labeled material synthesized according to Davies, Vanaman, and Stark (51). A solution of carbamylphosphate (4.8 mM) was incubated for 24 hours at 21°. Samples were withdrawn at intervals and carbamylphosphate quantitated by enzymatic conversion to carbamylaspartate using a large excess of aspartate transcarbamylase (51). The half-time of hydrolysis of carbamylphosphate under these conditions was 20 hours.

Stability of...
Table I

Molecular weights of aspartate transcarbamylase, its subunits and polypeptide chains

<table>
<thead>
<tr>
<th>Method</th>
<th>Aspartate transcarbamylase</th>
<th>Catalytic subunit</th>
<th>Regulatory subunit</th>
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<td>Native conditions</td>
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<tr>
<td>Sedimentation equilibrium</td>
<td>300,000</td>
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<td>28,000-30,000</td>
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<tr>
<td>Sephadex G-200 gel filtration</td>
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<td>96,000-110,000</td>
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<tr>
<td>Sucrose gradients</td>
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<td>100,000</td>
<td>28,000-35,000</td>
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Denaturing conditions

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<th>Guanidine hydrochloride</th>
<th>Sedimentation equilibrium</th>
<th>From [y]</th>
<th>From S and [y]</th>
<th>Sodium dodecyl sulfate</th>
<th>Sephadex G-200 gel filtration</th>
<th>Polyacrylamide gel electrophoresis</th>
<th>Chemical analysis</th>
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<tr>
<td></td>
<td></td>
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<td>32,000</td>
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</tr>
<tr>
<td></td>
<td></td>
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<td>19,300</td>
<td></td>
<td>16,000-19,000</td>
<td>16,000-18,000</td>
<td>17,000*</td>
</tr>
</tbody>
</table>

Carboxy-terminal analysis | 33,000
Amino acid sequence | (33,000)

* Data were taken from Reference 7.

See footnote 9 of text.

Aspartate transcarbamylase during prolonged periods of time at room temperature was tested in the same buffer. The enzyme was characterized at intervals by kinetic analysis, by sedimentation velocity centrifugation, and by acrylamide gel electrophoresis in sodium dodecyl sulfate. According to these criteria, the enzyme was unaffected by dialysis for at least 24 hours at 21°C.

**RESULTS**

**Molecular Weights of Regulatory and Catalytic Polypeptide Chains**

**Denaturation in Guanidine Hydrochloride**—Four different techniques—sedimentation equilibrium, sedimentation velocity, viscosity, and gel filtration—were used to determine the molecular weights of the isolated polypeptide chains in concentrated guanidine hydrochloride solutions (Table I).

The analyses of high speed sedimentation equilibrium experiments indicated that samples of isolated catalytic and regulatory subunits were homogeneous (Fig. 1). The molecular weight of the C chain was calculated to be 34,000 ± 1,500 in 8 M guanidinium hydrochloride (three experiments) and 34,000 ± 1,500 in 6 M guanidinium hydrochloride (two experiments). When the R subunit was centrifuged in 8 M guanidinium hydrochloride at a speed of 35,000 rpm, meniscus depletion was incomplete. In this case, the molecular weight obtained without corrections was of the order of 22,000. At higher speeds (42,040 rpm) the meniscus was fully depleted, and a value of 17,200 was obtained. The observed values extrapolate to intrinsic viscosities of 32,000 cc per g for the C chain and 22,300 cc per g for the R chain. Using the empirical expression relating the intrinsic viscosity and the chain length of fully denatured proteins (27), we calculated masses of 34,000 and 19,000 daltons for the catalytic and regulatory chains, respectively.

* These molecular weights were calculated using the partial specific volume determined from the amino acid composition. If a value of θ lower by 1% was used for the conversion of Mapp to the molecular weight of the catalytic and regulatory chains, values of 32,300 and 18,300 were obtained (cf. “Methods”).
Comparator x-Coordinate, mm (R)

Comparator x-Coordinate, mm (C)

Fringe Displacement on Comparator in mm

Lower Meniscus

C-Chain

R-Chain

Fig. 1. Sedimentation equilibrium analysis at 20° of the catalytic (C) chain in 6 M guanidine hydrochloride, 0.2 M β-mercaptoethanol, and the regulatory (R) polypeptide chain in 8 M guanidine hydrochloride, 0.5 M β-mercaptoethanol. Protein concentrations were 0.2 mg per ml. Rotor speeds were 35,600 rpm for the C and 42,040 rpm for the R chain. Fringe displacements are plotted as a function of the comparator x-Coordinates which are indicated for the catalytic chain at the bottom and for the regulatory chain at the top of the graph.

Fig. 2. The relationship between the reduced viscosities and protein concentrations in 6 M guanidine hydrochloride, 0.1 M β-mercaptoethanol, and 50 mM Tris-HCl, pH 7.5, at 25° for bovine serum albumin (a), catalytic polypeptide chain (b), and regulatory chain (c).

Fig. 3. The relationship between the reduced viscosities and protein concentrations in 6 M guanidine hydrochloride, 0.1 M β-mercaptoethanol, and 50 mM Tris-HCl, pH 7.5, at 25° for bovine serum albumin (a), catalytic polypeptide chain (b), and regulatory chain (c).

Denduration in Sodium Dodecyl Sulfate—Two different methods were used in this solvent: gel filtration on Sephadex G-200 and electrophoresis on polyacrylamide gels. Molecular weight determinations with these methods rely on the assumptions that proteins are fully dissociated into their polypeptide chains; that they bind equivalent amounts of the denaturant; and that different polypeptide chains assume a similar conformation. Sodium dodecyl sulfate has been shown to meet these conditions. It is an effective dissociating agent (16), and different proteins bind identical amounts of it (54). That polypeptide chains behave as though their apparent size is only determined by their molecular weight has been empirically demonstrated for polyacrylamide gel electrophoresis (16) and gel filtration (55). Two components of aspartate transcarbamylase were observed on a calibrated Sephadex G-200 column in 1% sodium dodecyl sulfate. They were identified as C and R chain by amino acid analysis and polyacrylamide gel electrophoresis in sodium dodecyl sulfate (see below). As seen in Fig. 3b, their respective molecular weights are in the range of 31,000 to 34,000 and 16,000 to 19,000. When native enzyme was subjected to electrophoresis on 10% polyacrylamide gels in 0.1% sodium dodecyl sulfate, two protein components were observed as well (7). The faster moving one corresponded to the regulatory, the slower moving one to the catalytic polypeptide chain. This conclusion, in turn, was based on experiments with isolated subunits (Fig. 4), as well as on amino acid analyses of the proteins eluted from these gels. The mobilities of the bands corresponded to molecular weights of 17,000 ± 1,000 for the R chain and 33,000 ± 1,000 for the C chain, in agreement with our previous results (7, 16). The calibration curve of sodium dodecyl sulfate gels (Fig. 3c) and curves obtained from gel filtration data are juxtaposed to illustrate the excellent agreement between the different methods.

It has recently been shown that some "pure" proteins can be contaminated with trace amounts of proteolytic enzymes, and that such impurities may cause misleading bands in sodium dodecyl sulfate gel electrophoresis, if no precautions are taken (56). We therefore checked the band patterns of the enzyme after extensive carboxymethylation in 6 M guanidine hydrochloride, as well as after oxidation with performic acid or kreat-
Fig. 3. Determination of the molecular weights of the polypeptide chains of aspartate transcarbamylase (ATCase). The calibration curves are those for gel filtration of carboxymethylated proteins in 6 M guanidine hydrochloride on a 6% agarose column (a), gel filtration of performic acid-oxidized proteins in 1% sodium dodecyl sulfate on Sephadex G-200 (b), and 10% polyacrylamide gel electrophoresis in sodium dodecyl sulfate (c). The distribution coefficients ($K_d$) and the electrophoretic mobilities are plotted versus the logarithms of molecular weights. The shaded areas indicate the range of the molecular weights for the catalytic and regulatory polypeptide chains.

Fig. 4. Separation and identification of the polypeptide chains of aspartate transcarbamylase by electrophoresis in 0.1% sodium dodecyl sulfate on 10% polyacrylamide gels. a, full enzyme; b, isolated catalytic (C) subunit; c, isolated regulatory (R) subunit; d, catalytic subunit obtained by heat treatment of aspartate transcarbamylase at low ionic strength. Electrophoretic migration is from top to bottom.

The molar ratio of the two polypeptide chains in the enzyme can be directly evaluated from stained gels (44). The ratio of the amount of protein present in the two bands was estimated by comparing the peak areas obtained after scanning the gels. In six different experiments, ratios between 1.9 and 2.1 were obtained. This distribution indicated that 66 to 68% of the total mass corresponded to C chains, whereas 34 to 32% could be assigned to R chains. This result is in agreement with the report of Gerhart and Schachman (8), who have derived a similar ratio from ultracentrifugal studies. Since the C chain has a mass twice that of the R chain, the two polypeptides must be present in the native molecule in equimolar amounts.

Molecular Weight of Native Aspartate Transcarbamylase and Its Subunits

The values for the molecular weights of the polypeptide chains were at variance with those reported earlier (5, 6), it was therefore necessary to reinvestigate the molecular weights of the native enzyme and the isolated subunits (Table I). Sedimentation equilibrium and sucrose gradient centrifugation as well as gel filtration chromatography were employed. The use of these techniques at very low protein concentrations allowed us to assess the presence or absence of association-dissociation equilibria.

Native Enzyme—High speed sedimentation equilibrium analysis of four different enzyme preparations showed that aspartate transcarbamylase is homogeneous and has a mass of 290,000...
I I I I I I

Fig. 5. Determination of molecular weights of typical globular proteins by gel filtration on Sephadex G-200 in phosphate buffer (40 mM, 0.1 M NaCl, pH 7.2) at 4°C. Molecular weights are plotted as a function of the distribution coefficients ($K_d$) of standard proteins. $K_d$ values of aspartate transcarbamylase (ATCase) and its subunits are indicated by arrows. Mb, (myoglobin); BCAB, (bovine carbonic anhydrase).

Fig. 6. Sedimentation equilibrium analysis of the catalytic subunit. High speed centrifugation at 16,200 rpm was performed in an AN-D rotor with a protein sample (0.2 mg per ml) in phosphate buffer (40 mM potassium phosphate, 0.1 M potassium chloride, 2 mM β-mercaptoethanol, 0.2 mM EDTA). The Fringe Displacement (indicated on the right-hand side of the graph) is plotted versus the Comparator x-Coordinate (top of graph). Low speed analysis at 7200 rpm was performed in an AN-G rotor with a protein sample (0.08 mg per ml) in the same buffer from which β-mercaptoethanol was omitted. The optical density (OD) at 230 nm is plotted versus the square of the radial distance, $r^2$, from the axis of rotation. Experiments were performed at 20°C.

to 310,000 daltons. The sedimentation coefficient in the range of 1 to 10 mg per ml was measured in the analytical ultracentrifuge, and a value of $s_{20,w} = 11.7$ S was obtained. To test the stability of the enzyme at very low concentrations, its sedimentation behavior was studied by sucrose gradient centrifugation. The $s$ value in the concentration range of 0.05 to 1 mg per ml (in the peak fraction) did not change appreciably and was always between 11.2 and 11.6 S. From this value and the sedimentation behavior of well characterized proteins, centrifuged in parallel gradients (25), we calculated an approximate molecular weight of 300,000 for aspartate transcarbamylase. Our results thus fully confirm those originally reported by Gerhart and Schachman (2) and Schachman and Edelstein (22) and strongly indicate the absence of an association-dissociation equilibrium.

We also studied the molecular weight of native enzyme by gel filtration. Three different samples, yielding concentrations of 5 to 250 μg per ml in the peak tubes, were chromatographed on a calibrated Sephadex G-200 column. The optical density profile of the eluate coincided with the activity profile, and the catalytic activity was inhibited by CTP. From the concentration-independent distribution coefficient of 0.22, a molecular weight of 300,000 to 310,000 was estimated from the calibration curve (Fig. 5). This procedure was justified since it was known from viscosity measurements of the native enzyme that the protein has a spherical shape (2). However, to check whether the enzyme also behaves as a typical globular protein in gel filtration, we determined the frictional ratio from the elution data and the molecular weight derived from sedimentation equilibrium (cf. “Experimental Procedures”). The value of 1.28 confirms the spherical shape and is in good agreement with the value of 1.32, obtained directly from the molecular weight (from sedimentation equilibrium) and the sedimentation coefficient.

Subunits of Aspartate Transcarbamylase—A molecular weight of 100,000 ± 6,200 (four experiments) was obtained for the C subunit by high speed sedimentation equilibrium studies. The plots of the logarithms of fringe displacement versus the square of the radial distance were linear to the bottom of the cell, indicating homogeneity of the protein (Fig. 6). Low speed sedimentation equilibrium at concentrations of 30, 85, and 150 μg per ml in an analytical ultracentrifuge equipped with a photoelectric scanning absorption system yielded a molecular weight of 99,000 ± 5,500 (Fig. 6). The sedimentation coefficient, measured at 80 μg per ml in this centrifuge, was 5.95 S. In sucrose gradient centrifugation of HMB dissociated enzyme, the C subunit (60 μg per ml in the peak fraction) migrated with a coefficient of 5.8 S. From this value, and assuming the protein
to be globular, an "apparent" molecular weight of 100,000 could be assigned to the catalytic subunit also by this method (25). The results obtained at low protein concentrations indicated the absence of an association-dissociation equilibrium. Gel filtration of the C subunit on Sephadex G-200 was performed in duplicate as described above for aspartate transcarbamylase. The concentrations of the isolated subunit in the peak tubes were 0.3 and 0.5 mg per ml. From the distribution coefficient of 0.42, a molecular weight of 96,000 to 110,000 was derived. To check whether the C subunit behaves as a globular protein during gel filtration, we calculated the frictional ratio from the elution position and the molecular weight determined by sedimentation equilibrium. The value obtained (1.24) indicates a spherical shape for the subunit, and is in excellent agreement with the one obtained directly from the sedimentation data (1.25).

Heat treatment of aspartate transcarbamylase (14) dissociated the enzyme into a soluble, catalytically active component and a precipitate which, according to polycrylamide gel electrophoresis in sodium dodecyl sulfate, consists predominantly of R chains. The soluble fraction contained a protein composed only of C polypeptide chains, as shown by gel electrophoresis (Fig. 4d) and amino acid analysis. This subunit sedimented as a single, symmetrical peak in the analytical ultracentrifuge (Fig. 7c). Its sedimentation coefficient (5.8 S), molecular weight (determined by sedimentation equilibrium), and kinetic properties were identical with those of the catalytic subunit, reactivated after isolation from HMB-dissociated enzyme. Treatment of the heat-dissociated C subunit with the mercurial did not affect its physical properties.

The regulatory subunit was examined by zone centrifugation and by gel filtration on Sephadex G-200. Calibration of sucrose gradients with cytochrome c and hemoglobin as standards gave an estimate of $s_{20,w}$ of 3.0 S for the R subunit over a concentration range of 0.03 to 1.0 mg per ml in the peak tube. If one assumes that the subunit is globular, this sedimentation coefficient corresponds to an "apparent" molecular weight of 28,000 to 35,000 (26). When the R subunit was chromatographed on Sephadex G-200 (0.2 mg per ml in the peak tube), it migrated with a distribution coefficient corresponding to a molecular weight of 28,000 to 30,000 (Fig. 5). We did not investigate a possible association-dissociation behavior of this subunit (4) further, but concluded from the molecular weights, obtained for the subunit and its polypeptide chain, that the isolated R subunit must exist predominantly as a dimer.

**Number and State of Half-cystine Residues**

Titration with 5,5'-dithiobis(2-nitrobenzoic acid) in 6 M guanidine hydrochloride yielded 29 to 31 moles of cysteine residues reacting in aspartate transcarbamylase (molecular weight 300,000). After oxidation of the enzyme by performic acid, amino acid analysis revealed a value for the cysteic acid content identical to that obtained by cysteine titration. These results excluded the presence of disulfide bonds in the native enzyme. Amino acid analysis after performic acid oxidation of the isolated subunits demonstrated the presence of 1 residue of cysteic acid per C chain (molecular weight 33,500) and 4 residues per R chain (molecular weight 17,000). The former result was confirmed by 5,5'-dithiobis(2-nitrobenzoic acid) titration of the C subunit in 6 M guanidine hydrochloride, and is further supported by preliminary sequence analysis.* The value of 4 cysteine residues per R chain was in agreement with the amino acid sequence of this polypeptide chain (7). The total number of sulfhydryl groups in aspartate transcarbamylase is accounted for, if we assume the presence of six catalytic and six regulatory polypeptide chains.

**Renaturation of Aspartate Transcarbamylase and Catalytic Subunit**

Aspartate transcarbamylase and the catalytic subunit were denatured in guanidine hydrochloride (6 M) or in sodium dodecyl sulfate (5%) in the presence of β-mercaptoethanol. From both solvents, the proteins could be reactivated in good yields. Renaturation from guanidine hydrochloride (70% recovery) was achieved by dilution. Sodium dodecyl sulfate was removed from the protein by anion exchange chromatography on Dowex-1 in the presence of 6 M urea prior to renaturation by dilution (30% recovery). The details of these procedures are described under "Experimental Procedures." Renatured catalytic subunit showed the same kinetic properties, sedimentation coefficient, and electrophoretic mobility in sodium dodecyl sulfate gels as the untreated material. Reactivated aspartate transcarbamylase was also shown to be identical with its untreated counterpart. Sedimentation velocity analysis of the renaturation products of full enzyme showed that in both preparations the boundary of the predominant component was sharp and moved with a sedimentation coefficient of 11.5 S (Fig. 7, e and f). The faster moving material had a sedimentation coefficient of approximately 16 S. It was similar to the small amount of higher aggregate which also appeared after reconstitution of HMB-dissociated enzyme (2) (Fig. 7d). The slowly moving boundary had a value of approximately 3 S. Sodium dodecyl sulfate gel electrophoresis of the latter component, isolated from sucrose gradient centrifugation, revealed that it consisted of regulatory polypeptide chains. To characterize the renatured enzyme further, we purified the 11.5 S component by sucrose gradient centrifugation. Kinetic analysis of this material revealed that the specific activity, cooperative behavior, and CTP inhibition were similar to those observed in native aspartate transcarbamylase. Sodium dodecyl sulfate gel electrophoresis revealed the band pattern typical for this enzyme. The renatured protein contained zinc (see below), and was crystallized, yielding the trigonal crystal form which was also obtained with native aspartate transcarbamylase.**

**Determination of Number of Binding Sites for Succinate and Metal Ions**

Succinate Binding—Binding to the catalytic subunit was examined at 4°C by the technique developed by Colowick and Womack (49). The conditions and the results are presented in the Scatchard plot (57) of Fig. 8. For three different experiments, straight lines representing the best fits were calculated by the least square method. The average of these results was

* Cleavage of catalytic subunit at cysteine residues according to the procedure of Vanaman and Stark (58) yields two fragments with molecular weights of 5,500 and 27,500 (K. Weber, unpublished observation). The sum of the weights of these fragments confirms the molecular weight of 33,000 daltons per C chain.

** D. C. Wiley, D. R. Evans, S. G. Warren, and W. N. Lipscomb, personal communication. Recently, they have obtained a crystal form of a nitrotyrosyl derivative of aspartate transcarbamylase which contains both a 3-fold and a 2-fold symmetry axis.
FIG. 7. Schlieren patterns of aspartate transcarbamylase and its subunits. a, native enzyme ($s_{w0} = 11.7 S$); b, the dissociated subunits; and c, native enzyme reconstituted after HMB dissociation. The enzyme used in these three runs contained 6 g atoms of cadmium per 300,000 g of enzyme rather than zinc, and was obtained by the replacement of ZnSO₄ in the growth medium by CdCl₂. e, catalytic subunit obtained by heat treatment of native enzyme in a low ionic strength buffer. f, aspartate transcarbamylase renatured from 6 M guanidine hydrochloride, 0.1 M β-mercaptoethanol; f, enzyme reactivated from 5% sodium dodecyl sulfate and 1% β-mercaptoethanol. Pictures were taken 24 min after reaching a speed of 50,740 rpm, except for b, which was photographed 24 min later. Sedimentation was from left to right.

3 binding sites per catalytic subunit (standard deviation of ± 0.3) and a dissociation constant at this temperature of $1.1 \times 10^{-4}$ M. No indication of cooperative binding of succinate to the catalytic subunit was observed.

Metal Content of Aspartate Transcarbamylase—The presence of zinc in the growth medium (12) for the uracil-requiring strain of E. coli is important for good yields of aspartate transcarbamylase. Dr. J. Gerhart has kindly informed us about this requirement and the fact that native enzyme contains zinc ions. For our study of the structure of this enzyme, it was important to determine the stoichiometric relation between metal ions and enzyme. We therefore measured the zinc content of this protein in five different enzyme preparations. The values obtained varied between 5.3 and 6.4 g atoms of zinc per mole of protein, with a mean of 5.72 and a standard deviation of ± 0.23 (20 determinations). These results are in agreement with the values found by Nelbach and Gerhart. The metal ions are bound very tightly to the protein, since prolonged incubations with o-phenanthroline or hydroxyquinoline sulfonic acid, followed by the removal of the chelating agents by gel filtration, had no effect on the metal content (5.9 and 5.7 g atoms of zinc per 300,000 g of protein). No other transition metals could be detected in the enzyme. Catalytic subunit, prepared by HMB-dissociation and subsequently isolated by anion exchange chromatography, contained no zinc. Heat-prepared subunit was also metal free after gel filtration through Sephadex G-25.

Both methods of preparation yielded catalytic subunit which was fully active; zinc is therefore not required for enzymatic activity.

Zinc could be replaced by cadmium in two ways. For the replacement in vivo, the uracil-requiring strain of E. coli was grown in a medium very similar to that described by Gerhart and Holoubek (12), except that CdCl₂ replaced ZnSO₄, and deionized distilled water was used. The enzyme was purified by the standard procedure. The metal content of the pure enzyme was 5.4 to 5.8 g atoms of cadmium per mole of enzyme, and only trace amounts of zinc were found. The kinetic properties of the cadmium enzyme were identical to those of zinc aspartate transcarbamylase, and both enzymes gave the same band pattern on polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The sedimentation coefficient of the cadmium enzyme was 11.7 S (Fig. 7a); it could be dissociated by HMB (Fig. 7b) and reconstituted by β-mercaptoethanol (Fig. 7d).

These are chromium, manganese, iron, cobalt, nickel, copper, cadmium, and mercury. Alkaline earths (calcium and magnesium) were found in varying amounts, but did not exceed 1 g atom per mole of protein.
The enzyme was crystallized, yielding the trigonal crystal form also obtained with zinc enzyme.  

Cadmium aspartate transcarbamylase could also be prepared from purified zinc enzyme by HMB-dissociation and metal replacement in vitro. EDTA (3 mM) was added to HMB-dissociated zinc enzyme and excess mercurial and zinc (as a ZnEDTA complex) were removed by gel filtration through Sephadex C 25. The fractions containing protein were pooled and an amount of CdCl₂ equimolar to the concentration of metal binding sites was added. Reconstitution was achieved by addition of β-mercaptoethanol (2 mM). The reconstituted enzyme contained 5.5 to 5.8 g atoms of cadmium per mole aspartate transcarbamylase, and only trace amounts of zinc. The experiment could also be performed in the reverse direction. Native cadmium enzyme, obtained by incorporation of this metal in vivo, was dissociated by HMB, and cadmium was replaced by zinc as described above. This reconstituted in vitro zinc enzyme could not be distinguished from the native enzyme.

**DISCUSSION**

The molecular weights of the two different polypeptide chains of aspartate transcarbamylase have been determined by several independent methods. Three well founded and generally accepted techniques (Table I) were used in a solvent system (guanidine hydrochloride, β-mercaptoethanol) in which the conformation of polypeptide chains is well understood (27). Yet these three methods can still be subject to certain errors. A decrease of the partial specific volume of a protein at high temperatures would be expected to decrease the molecular weight calculated from sedimentation equilibrium analysis, as well as the adjustment of sedimentation coefficients to standard conditions. The calculation of the molecular weights from the intrinsic viscosities of polypeptide chains in guanidine hydrochloride, while based on a well studied empirical relation (27), cannot be expected to yield values correct to within less than 5 to 10%. In spite of these potential uncertainties, the molecular weights obtained by these methods are in close agreement with each other. Similar values, and a comparable accuracy, were also reached by gel filtration in sodium dodecyl sulfate and guanidine hydrochloride, and by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The reliability of the latter two procedures has been amply demonstrated by Fish et al. (41) and by Weber and Osborn (16). In our hands they are useful tools for the determination of polypeptide chain molecular weights, since they require small amounts of material and are easily and rapidly performed.

The molecular weight of the native enzyme was investigated by ultracentrifugation and gel filtration on Sephadex G-200. The methods used (Table I) yielded a molecular weight of 300,000 and demonstrated the absence of an association-dissociation equilibrium, in agreement with previous results reported by Gerhart and Schachman (2), and Schachman and Edelstein (22). As a further check of our own results, we calculated the frictional ratio of the protein in two independent ways. The close agreement between the two values is a good indication for the reliability of the experimental data from which they were derived. The stoichiometric relation between catalytic and regulatory chains can be calculated from the distribution of sulfhydril groups in the two chains, or directly from the mass ratios observed in polyacrylamide gel electrophoresis. Only a distribution of 6 catalytic and 6 regulatory chains per enzyme molecule can account for all experimental results. This conclusion is supported by the amino acid compositions of the enzyme and its subunits. In agreement with a structure (RC)₆ is the metal content of aspartate transcarbamylase. We showed that 1 molecule of enzyme contains 6 tightly bound zinc ions, which are not required for catalytic activity. Zinc can be replaced by cadmium both in vivo and in vitro, and this exchange does not affect the kinetic and physical properties of the enzyme.

The agreement of the results of 5,5′-dithiobis(2-nitrobenzoate) titrations in guanidine hydrochloride solution with the cysteic acid content of enzyme which was oxidized with performic acid, indicates that there are no disulfide linkages in aspartate transcarbamylase. The polypeptide chains must therefore be held together solely by noncovalent forces, a result which was also arrived at using polyacrylamide gel electrophoresis in sodium dodecyl sulfate in the presence of HMB.

The molecular weight and the stability of the catalytic subunit were examined as described for the native enzyme. The results of these studies confirm that the C subunit has a molecular weight of 100,000, and show that it has no noticeable tendency to dissociate or aggregate in the concentration range examined. From these results and the molecular weight of the C chain, we can conclude that this catalytically active subunit is a stable trimer. It is therefore reasonable to assume (1) that it contains a 3-fold axis of symmetry. However, stable proteins with an odd number of polypeptide chains are thought to be disfavored by selection (10), and odd-numbered symmetry has rarely been found in proteins. Yet, a 5-fold symmetry axis has recently been proposed for arginine decarboxylase on the basis of both chemical evidence and electron microscopy (50). Since the catalytic subunit appeared to be a trimer, it was important to document its stability further. Two lines of evidence can be cited to support this. (a) When native enzyme is incubated at high temperature, the molecule dissociates, releasing into solution an enzymatically active subunit in high yield. The characteristics of this subunit are identical with those of the active C subunit, isolated after HMB dissociation of native enzyme. That an identical structure can be obtained by two different treatments demonstrates the stability of the catalytic subunit. (b) Renaturation of C subunit from 6% guanidine hydrochloride or 1% sodium dodecyl sulfate can be achieved with high recovery. The reactivated subunits are indistinguishable from the active subunit obtained by HMB dissociation of native aspartate transcarbamylase. That fully denatured catalytic polypeptide chains can regain their tertiary conformation, and assume a quaternary structure identical with the one obtained by dissociation of the full enzyme, indicates once again that this conformation is a stable and “closed” one.

Given that the catalytic subunit is a trimer, it is important to decide whether its trimeric structure is preserved in native aspartate transcarbamylase. This would convey the 3-fold axis of symmetry also to the native enzyme. Several lines of evidence support this hypothesis. (a) It has been discussed that heat treatment of aspartate transcarbamylase gives a subunit which is identical with that obtained by HMB dissociation. That two different treatments yield a unique submolecular entity indicates that this entity is a direct dissociation product of the enzyme rather than a product of a secondary reaction. (b) The renaturation of C subunit, discussed above, must be considered in connection with the finding that C chains can also renature to give native aspartate transcarbamylase, if
regulatory chains are renatured along with them. The specificity of association of polypeptide chains in an oligomer is very high (1), and it therefore seems unlikely that a polypeptide chain has two distinct binding sites which can lead to two different, but equally stable, oligomeric structures. We propose that the geometric disposition of the catalytic polypeptide chains in native aspartate transcarbamylase is identical with that in the C subunit, and that they are indeed present in the native enzyme as two trimers. (c) This conclusion is further supported by recent experimental evidence of Meighen, Pigiet, and Schachman (60). Reconstitution of a mixture of native and partially succinylated C subunits in the presence of unaltered R subunits yielded three species of aspartate transcarbamylase which could be separated by electrophoresis. This result suggests that the C subunits are indeed incorporated as unaltered trimers into the enzyme during reconstitution. If a rearrangement of C chains were to occur during this process, seven rather than three species would have been observed.

In summary, the same structure, the native enzyme, can be formed from two levels of organization, either from the polypeptide chains or from the subunits. The different reactions can be represented by Scheme I. The conclusion that the six C chains are present in native aspartate transcarbamylase in two trimers allows us to propose a three-dimensional arrangement of the polypeptide chains in this protein. Formally, we can consider the enzyme to consist of six protomers (RC) and can represent its structure as (RC)_6. It is a widely accepted assumption that in a stable oligomeric protein the protomers occupy spatially equivalent positions (1). This, in turn, implies that the molecule possesses axial symmetry. A priori, a hexamer can assume two symmetrical dispositions which account for the experimental finding that it is a compact, stable, closed system with no tendency for further association: a planar, hexagonal ring (C_6), or a configuration with one 3-fold and three 2-fold axes of symmetry (D_32). The former structure, however, is unlikely, since the domains of bonding between the six catalytic polypeptide chains would have to be identical. Preferential dissociation into trimers would therefore not be expected. A rapid cleavage into individual polypeptide chains, followed by reassociation to trimeric subunits, can be ruled out on the basis of the experimental evidence of Meighen et al. (60), which was discussed above. On the other hand, 32 symmetry does account for the observed dissociation products of aspartate transcarbamylase and is consistent with our conclusion that the C subunits exist as trimers in the native enzyme. In such an arrangement, the two catalytic trimers would be stacked on top of each other in a structure with three 2-fold axes of symmetry. The R chains can then be arranged in two ways without affecting this symmetry: they can be intercalated as dimers between the catalytic subunits, or they can be arranged as six monomeric units on the outside of the two trimers. In the latter arrangement, the two C subunits would be in direct contact with each other. We tend to favor the former alternative for the reason that R subunits occur predominantly as dimers after HMB dissociation. Presently, we do not have direct evidence that the dimeric structure of the R subunit is actually preserved in the native enzyme. However, one can argue that only one type of bonding domain (C—R contacts) has to be cleaved by HMB or heat dissociation to yield catalytic trimers, if the R chains are located between the C subunits. Were they situated individually on the outside of the molecule, the contacts between the then contiguous C subunits would have to be cleaved as well to account for the dissociation products. Though both arguments are not conclusive, and render the structure with intercalated R subunits only more likely, they allow us to propose as a working hypothesis the geometrical arrangement of the polypeptide chains which is shown in Fig. 9. It should be pointed out that the representation of the catalytic and the regulatory chains in exact vertical alignment has been chosen solely for the purpose of a clearer diagram, although staggering of the different units is just as likely. Conclusive evidence for the three dimensional arrangement will, of course, have to await detailed x-ray crystallographic analysis. At present, the data of Wiley and Lipscomb (9) support our model. They found a 3-fold axis of symmetry in a trigonal crystal and a 2-fold axis in a tetragonal crystal of the aspartate transcarbamylase molecule. These data are in agreement with a (RC)_6 structure, but on their basis, a distinction between hexagonal and 32 symmetry is not possible.

How can the results which originally led to the tetrameric
model be explained? It is not difficult to interpret these data in retrospect. Amino-terminal analyses (5, 6) can suffer from incomplete recoveries. Fingerprint analysis (6) after tryptic digestion can be obscured by incomplete or nonspecific enzymatic cleavages. Both errors would lead to the assumption of too high molecular weights. The determination of the molecular weight in urea solution (5) was performed at slightly low centrifugal speeds, so that the meniscus was probably not fully depleted.

Of particular interest is the number of binding sites in this enzyme. Since a number lower than that of the catalytic polypeptide chain present would have important implications on the allosteric mechanism of aspartate transcarbamylase, it was necessary to determine whether the apparent discrepancy between earlier binding data and the subunit structure presented here could be the result of the methods employed in the previous studies. Inactivation of the enzyme, or decomposition of the ligands, could have affected earlier investigations. Alternatively, the effect of the Donnan equilibrium might have been underestimated. We have ruled out the first alternative (cf. “Experimental Procedures”), but believe that the Donnan effect can account for the lower number of sites (32) obtained by Changeux, Gerhart, and Schachman (4). We have therefore re-investigated the binding of the substrate analogue, succinate, to the catalytic subunit by the dialysis rate method of Coldowick and Womack (49) which depends on reaching a steady state rather than an equilibrium. This technique yielded three binding sites for succinate in the catalytic subunit. Hammes, Porter, and Wu (61) have independently demonstrated by the method of continuous variation that this subunit also binds three equivalents of carboxymethylamino-CPA. Both of these techniques are independent of a possible unequal distribution of electrolytes. Thus the two complementary ligands have the same number of sites in the isolated catalytic subunit. This stoichiometry, as well as the result of Hammes et al. (61) that aspartate transcarbamylase binds six equivalents of bromo-CPA in agreement with the subunit structure presented here. It should, however, be pointed out that no inference can be made from these results as to the binding properties of the substrates (or their analogues) in the undissociated enzyme. Experiments to determine the binding parameters of native aspartate transcarbamylase are currently being performed, since this information is important for the understanding of its allosteric mechanism.

Acknowledgments—We are grateful to Dr. J. Gerhart for communicating his results prior to publication; to Dr. F. Quiocho for making available to us his procedure for the enzyme assay; to Drs. M. Young and R. Weil for their collaboration in ultracentrifugal studies with the photoelectric scanning absorption system; and to Dr. G. Guidotti for many stimulating discussions. We are indebted to E. Bloomquist for excellent technical assistance and to R. Rosenbusch for her help in the preparation of the manuscript. The New England Enzyme Center has provided the facilities for large scale preparation of enzyme.

After this manuscript had been completed, two reports (62, 63) concerning the binding of CTP in aspartate transcarbamylase were published. Both papers postulate two classes of binding sites for this effector, but disagree among themselves and with the data of Hammes et al. (61) on the number and significance of the different sites. We feel that more data are required to clarify these results.

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