Studies on Adenosine Triphosphate Transphosphorylases. V. Studies on the Polypeptide Chains of the Crystalline Adenosine Triphosphate–Creatine Transphosphorylase from Rabbit Skeletal Muscle*

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ABSTRACT: An investigation on the subunit structure of crystalline adenosine triphosphate–creatine transphosphorylase from rabbit skeletal muscle has been made by both physical and chemical means. A redetermination of the molecular weight of the native enzyme by sedimentation velocity, diffusion, and sedimentation equilibrium proved necessary to provide a more quantitative comparison for ultracentrifugal studies which were conducted in the presence of dissociating and denaturing solvents.

Physical measurements in urea or guanidine solutions (in the presence or absence of mercaptoethanol) coupled with quantitative chemical analyses on the lysine carboxyl-terminal groups and with analyses on the tryptic peptides, permit the conclusion that the enzyme molecule is composed of two noncovalently linked polypeptide chains with similar, if not identical, physical and chemical properties. Coincident with these studies, the physical effects of a few disruptive agents have been explored to define the conditions for dissociation and to provide an insight into the dissociation phenomenon.

The quantitative amino acid analyses (Noltmann *et al.*, 1962) of the crystalline ATP[1]–creatine transphosphorylase (Kuby *et al.*, 1954) provided the basis for a course of investigations on the relationship between the chemical and physical properties of this protein and laid the foundation for explorations into its amino acid sequence. Such studies will undoubtedly prove useful in the final chemical evaluation of the enzymatically catalyzed reaction.

This report is concerned primarily with an elucidation of the "subunit" structure of the enzyme protein by both physical and chemical means. During the course of these investigations, a reexploration of the physical properties of the native enzyme (*cf.* Noda *et al.*, 1954) proved necessary, to provide a more quantitative comparison for the ultracentrifugal studies which were conducted in the presence of dissociating and denaturing solvents. These data, then, when coupled with quantitative chemical analyses on the carboxyl-terminal groups and with qualitative analyses of the tryptic peptides, permit the conclusion that this enzyme is composed of two polypeptide chains of remarkable similarity in physical and chemical properties. Independently, Kaplan and his associates (Dawson *et al.*, 1965, 1967; Eppenberger *et al.*, 1967), from studies on the comparative enzymology of the ATP–creatine transphosphorylases from several sources (in particular, the enzymes from chicken muscle and brain), postulated that this enzyme is dimeric in structure, and this conclusion has been in agreement with several other types of studies (*e.g.*, Dance and Watts, 1962; Eveleigh and Thomson, 1965; Hooton and Watts, 1966).

In addition, and coincident with studies reported herein, the physical effects of a few dissociating agents have been carefully explored from the points of view of defining the conditions for dissociation and shedding some light on the dissociation phenomenon. Further, a quantitative reinvestigation of the carboxyl-terminal groups has confirmed the earlier observation (Olson and Kuby, 1964) that lysine is at the carboxyl terminus of the protein, but the data reported here provide a better correlation with the physical data. A preliminary report of these investigations has been presented (Yue *et al.*, 1967a).

Materials and Methods

Materials. ATP–creatine transphosphorylase was isolated from rabbit skeletal muscle according to the procedure of Kuby *et al.* (1954) and crystallized three

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[1] Abbreviations used: ATP, adenosine triphosphate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; BAEE, *N*-benzoyl-L-arginine ethyl ester; IAA, iodoacetic acid.

times according to the modifications described (Mahowald *et al.*, 1962a). This preparation (5D, 75 units/mg) was used for most of the studies reported here, except those indicated in the text (*i.e.*, in the case of older preparations, three-times crystallized 3D and 4D), and was stored as a crystalline suspension at −12° for the duration of these experiments. When required, aliquots of the crystalline suspensions were removed, the crystals were harvested by centrifugation (at −12°), and the enzyme was equilibrated by dialysis (at 3°) against the buffers used for studies on the native enzyme (see legends to the appropriate figures for details on the buffer compositions). Other treatments of the enzyme, *e.g.*, for the ultracentrifuge studies in denaturing solvents, for the chemical studies on terminal groups, and for the preparation of derivatives prior to tryptic digestion, are described below. Amino acid analyses (20-hr acid hydrolysates) of the preparation agreed with those reported earlier (Noltmann *et al.*, 1962).

Preliminary tests on the efficacy of the denaturing solvents as dissociating agents and the utility of the Rayleigh interferometric optics for measurements of the concentration distribution, in the several solvents at sedimentation equilibrium, were made on a few proteins which included: chromatographed bovine pancreatic ribonuclease A (Sigma Chemical Co., lot 63B-3560-7; 126 Kunitz units/mg), crystalline glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle (Calbiochem Inc., lot 35112, 46 units/mg), crystalline bovine plasma albumin (Armour, lot no. X 69508), and crystalline horse liver alcohol dehydrogenase (prepared by the procedure of Bonnichsen and Brink (1955), repeatedly crystallized from dilute ethanolic phosphate (pH 7) to remove traces of hemoglobin; dialyzed *vs.* 0.01 M pyrophosphate (pH 8), lyophilized, and stored at −10°).

Guanidine hydrochloride (Eastman, White Label) was twice recrystallized by dissolving in a minimum volume of absolute methanol (Anson, 1941) and concentrated by means of an all-glass rotary evaporator until crystallization (*cf.* Nozaki and Tanford, 1967). The dried guanidine hydrochloride was stored in a deep freeze and used within a month's period.

Urea (Mallinckrodt, analytical grade reagent) was deionized and crystallized from saturated aqueous solutions according to Benesch *et al.* (1954, 1955) (except that MB-3 mixed-bed resin (Mallinckrodt) was used for deionization, instead of MB-1). Sodium lauryl sulfate samples were obtained from Sigma (lots 16B-009C and S110B-69) (both proved to be identical in behavior).

β-Mercaptoethanol (Eastman) was redistilled *in vacuo*. Pyridine (Baker or Mallinckrodt, analytical grade reagent) was refluxed with phthalic anhydride and redistilled. Butanol, and other solvents employed, were purified primarily by distillation. All other analytical grade reagents were the best available commercial products and were used without further purification (except, in particular, those reagents described below under chemical studies). Twice-distilled deionized water was used in the preparation of all solutions (especially for the physical methods) and degassed by boiling, when necessary.

Physical Measurements. The preparation of protein solutions was made as follows. For the native enzyme, solutions were prepared by dissolving the crystals in the appropriate buffer, followed by dialysis at 3° against repeated changes of the same buffer for at least 24 hr; concentrations were determined spectrophotometrically, with an extinction coefficient $E_{280}^{1\%}$ 8.88 (after dilution in 0.05 M phosphate, pH 7.0) (Kuby *et al.*, 1962).

For those experiments with urea or guanidine hydrochloride, the following procedure was used, except in some special cases, which will be indicated in the text. The protein solutions, which had been equilibrated against buffers at pH 7.8, were dialyzed exhaustively at 3° against redistilled water, degassed by boiling. Aliquots of the salt-free solutions (whose protein concentrations were determined) were accurately pipetted into vials and lyophilized in desiccators attached to an all-glass apparatus (Mahowald *et al.*, 1962b). Freshly prepared and pH-adjusted urea or guanidine solutions (for their compositions and those of other components, see text) were added to the vials containing lyophilized salt-free protein samples to yield the desired protein concentrations after dissolution, and the capped vials were then allowed to stand at the desired temperature for at least 1 hr prior to measurement. (The times are indicated in the legends.) The protein-containing vials were kept tightly capped, except during the addition of the solvent and the removal of an aliquot for the ultracentrifuge cell, in order to prevent any change in volume (by evaporation; *e.g.*, see Reithel *et al.*, 1964). These precautions were necessary in order to balance the concentrations of guanidine in the solvent and solution sectors of the ultracentrifuge cell, when Rayleigh interferometric optics were employed. A small difference in concentration of the urea or guanidine between the sectors, at the concentrations employed, is readily detected interferometrically and can cause serious difficulties in interferometric analyses (see Schachman and Edelstein, 1966). The lyophilized protein samples were used shortly after lyophilization to minimize any increase in moisture. Dilution of the urea or guanidine concentrations in the solution sector compared to the solvent sector by moisture usually present in the lyophilized protein samples (as well as by changes in volume due to the partial specific volume contribution by the protein) proved to be well within the error of detection by the Rayleigh interferometric optics, at the protein concentrations employed in these investigations (see text).

Sedimentation Measurements. SEDIMENTATION VELOCITIES were measured at 3° (or 20°, where indicated in the text) in a Spinco Model E ultracentrifuge, equipped with an RTIC temperature control system and phase plate as schlieren diaphragm. The schlieren optical system was aligned according to Gropper (1964). Kodak metallographic (or in a few cases, spectroscopic type ID) plates were used to photograph

the schlieren patterns. Both single-sector cells of 12-mm optical path (especially for the native enzyme) and filled-Epon double-sector 12-mm cells (used primarily for denaturing conditions) were employed. Sedimentation coefficients at 3° and buffer ($s'_{3°,b}$) (or at 20°, *i.e.*, $s'_{20°,b}$) were calculated in the usual fashion from log X *vs.* t plots (Schachman, 1957) and converted to $s_{20°,w}$ values (Svedberg and Pedersen, 1940) with the use of measured values for the densities and viscosities of the buffers and denaturing solvents employed. For the protein solutions in denaturing solvents, the $\bar{V}$ calculated from the amino acid composition (0.735 cc/g) (Noltmann *et al.*, 1962) was used, whereas for the native enzyme the partial specific volumes extrapolated from the data of Noda *et al.* (1954) to zero concentrations at 3 or 20° were employed.

SEDIMENTATION EQUILIBRIUM experiments at 3° (or 20°) were made with a rotatable light source and with the use of Rayleigh interferometric optics (Richards and Schachman, 1959), and with the optics aligned also according to Gropper (1964). A slow stream of air was passed over the outside of the lower collimating lens to eliminate condensation of moisture during the centrifugation period (Yphantis, 1964). For these studies, a 12-mm double-sector synthetic boundary cell of the capillary type, with sapphire windows, was employed and Kodak IIG spectroscopic plates were used for recording the interferograms. The experimental procedure followed that described (Spinco Division Manual, 1964) with slight modifications (Yue *et al.*, 1967b). In the experiments with protein solutions containing high concentrations of denaturants (urea or guanidine), each sector was filled with the same volume (137 μl) to match the menisci, as discussed by Schachman and Edelstein (1966). To minimize any convective disturbances due to changes in temperature, the solutions, the cell, and the rotor were all equilibrated at 20°, and all manipulation times were reduced to a minimum, but a slow acceleration time was employed. To decrease the time required to reach sedimentation equilibrium, the rotor (AN-D or AN-E) was overspeeded for a calculated period of time followed by a reduction in speed to the desired equilibrium speed (Hexner *et al.*, 1961), which was selected to provide a three- to fourfold ratio in (C_b/C_m) (the concentration at the cell bottom to that at the meniscus (Svedberg and Pedersen, 1940)). The legends to the figures give the pertinent information. The apparent weight-average molecular weights at various radial positions in the cell, at equilibrium, were calculated from ln J_x *vs.* X^2 plots according to $M_{w,x} = [2RT/\{(1 - \bar{V}\rho)\omega^2\}] \cdot (d(\ln J_x)/dx^2)$ and the mean z-average molecular weight for the entire cell contents was calculated from the limiting slopes of the ln J_x *vs.* X^2 plots, at the cell bottom and the meniscus, with the use of the equation $\bar{M}_z = (M_{w,x_b}C_b - M_{w,x_m}C_m)/(C_b - C_m)$ (Svedberg and Pedersen, 1940). The calibration of the instrument (and meaning of the terms employed) has been described (Yue *et al.*, 1967b). As a further check on the interferometric procedure for sedimentation equilibrium analyses at high guanidine hydrochloride–mercaptoethanol concentrations, preliminary tests were made on the following protein systems, where in two cases dissociation would not be expected to occur and in two cases dissociation would be expected. In 4 M guanidine hydrochloride–0.1 M β-mercaptoethanol (pH 6.3) the apparent mean weight-average molecular weights (together with the values for the partial specific volumes selected for calculation) which were obtained for bovine pancreatic ribonuclease (at 4.0 mg/ml), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (at 3.5 mg/ml), horse liver alcohol dehydrogenase (at 2.5 mg/ml), and bovine plasma albumin (at 3.5 mg/ml) were, respectively, 13,600 (with $\bar{V} = 0.695$; Van Holde and Baldwin, 1958; Harrington and Schellman, 1956), 35,300 (with $\bar{V} = 0.739$; Taylor and Lowry, 1956), 42,900 (with $\bar{V} = 0.750$; Ehrenberg and Dalziel, 1958), and 65,400 (with $\bar{V} = 0.734$; Edsall (1953) (calculated from data of Stein and Moore, 1949) and Dayhoff *et al.*, 1952). These values for $\bar{M}_w$ may be compared with those conveniently summarized by Klotz (1967). In general, the most consistent values for $\bar{M}_w$, in 4 M guanidine solutions, were obtained with the use of the $\bar{V}$ calculated from the amino acid composition.

The interferometric technique of sedimentation equilibrium in highly dilute protein solutions and at relatively high speeds, according to Yphantis (1964), was also utilized in this study. Blank runs were made immediately after each experiment, with the same cell which had been cleaned without dismantling, in order to correct for residual optical inequalities between solution and solvent channels, as described by Yphantis (1964). (Care must be exercised with the use of this technique to ensure that oil and dust do not collect on the collimating lenses, and straight and parallel "air fringes" have been used as a qualitative index of cleanliness of the lenses.) Evaluation of molecular weight averages ($\bar{M}_n$, $\bar{M}_w$, and $\bar{M}_z$) was made according to the procedures of Yphantis (1964). A somewhat larger degree of uncertainty was obtained in estimations of $\bar{M}_w$ by this technique than by the more conventional sedimentation equilibrium procedures.

Diffusion. Free diffusion coefficients (D) were determined at 3.00° with the use of a Spinco Model H electrophoresis diffusion instrument with the light source filtered to yield 546 mμ. For all the studies reported here, both the modified 2- and 11-ml electrophoresis cells were employed and the experimental procedures as well as the method of boundary sharpening followed those described by Schachman (1957). Diffusion coefficients were calculated, for the most part, from photographs (on metallographic plates) of the Rayleigh interferograms. Computations were made by the method of Longsworth (1952) and the Tables of Probability Functions (1941). The measured values for the diffusion coefficients (D'_{meas}) were then corrected for the uncertainty in the "zero time" by means of the expression: $D'_{meas} = D(1 + (\Delta t/t'_{meas}))$, where D is then $D_{3°,b}$, for 3° and buffer (Longsworth, 1947). To convert $D_{3°,b}$ to "standard conditions" of $D_{20°,w}$, the Stokes–Einstein relation was employed

with the use of measured values of the viscosity. Weight-average molecular weights ($M_{s,D}$) from extrapolated data for diffusion and sedimentation velocity (see above), and from $\bar{V}$ data, were obtained from the Svedberg equation. Since Gosting and Morris (1949) have found slight inherent errors in the use of the Stokes–Einstein relation, calculations of $M_{s,D}$ were made as well from the $s^0_{3^\circ,b}$, $D^0_{3^\circ,b}$, and $\bar{V}^0_{3^\circ}$ sets of data. The calibration of this instrument has been described (Yue *et al.*, 1967b).

Chemical Studies. PREPARATION OF PROTEIN DERIVATIVES[2] FOR TRYPTIC DIGESTIONS. Although several derivatives of this protein have been studied, only three types of preparation are reported here to illustrate their tryptic digestibility (see below), when applied in particular to subunit analyses. (a) Performic acid oxidized protein was prepared (at −10°) from salt-free lyophilized protein (three-times-crystallized preparation 4D) by a procedure based on that of Hirs (1956), rendered free of peroxides, and lyophilized. Cysteic acid and methionine sulfone analyses performed on 20-hr 6 N HCl hydrolysates with a Spinco 120B (Moore *et al.*, 1958) gave 7.7 and 17.9 residues, respectively, per 83,600 g (uncor) (*cf.* Noltmann *et al.*, 1962). (b) *S*-Carboxymethylated protein prepared in 0.7% lauryl sulfate with $NaBH_4$ followed by alkylation with iodoacetic acid was carried out on salt-free lyophilized protein (4D) by a procedure based on Noltmann *et al.* (1962), freed of salt and reagents by dialysis, and lyophilized. Analyses revealed 6.9 residues (uncor) of *S*-carboxymethylcysteine/mole (*cf.* Noltmann *et al.*, 1962). (c) *S*-Carboxymethylated protein prepared in 8 M urea with mercaptoethanol followed by alkylation with iodoacetic acid was carried out on two salt-free lyophilized preparations (preparations 5D and 4D) under just slightly different conditions, by procedures based on that of Crestfield *et al.* (1963), followed by removal of excess reagents by exhaustive dialysis and lyophilization. Analyses of 20-hr acid hydrolysates of these preparations yielded 7.7 and 7.3 residues (uncor) of *S*-carboxymethylcysteine per 83,000 g of protein for preparation 5D and 4D, respectively. Moisture contents of the derivatives (a–c) were determined by drying to constant weight *in vacuo* over P_2O_5 at 74°.

TRYPSIN DIGESTION. These were carried out on weighed samples, with the use of an autotitrator (Radiometer TTT-1a, pHA 630Ta, SBR2C, and SBU1a), a temperature-controlled pH-Stat vessel under a nitrogen barrier, and standardized NaOH as titrant. The particular reaction mixture conditions employed for each of the preparations given above are described later on. When the system appeared to stabilize and the NaOH uptake decreased to an extremely slow rate, an aliquot of a freshly prepared solution of trypsin (in ice-cold 0.001 M HCl) was added to initiate the reaction; a second addition of trypsin was added after more than 50% of the total reaction was apparently complete and continued until the reaction rate appeared to approach the blank rate. The trypsin preparation (three-times crystallized, Worthington) used for the *S*-carboxymethylated protein treated with mercaptoethanol in 8 M urea had been treated with TPCK (Calbiochem) according to Kostka and Carpenter (1964) to inhibit possible traces of chymotryptic activity, and the resulting trypsin preparation assayed titrimetrically with BAEE as substrate. Aliquots for ninhydrin analyses (Troll and Cannan, 1953) were also removed from the pH-Stat vessel at periodic intervals to follow the reactions (but these are not reported here) and they qualitatively followed the course of the reaction given titrimetrically.

Aliquots of the final digests were used for peptide mapping (see below) and after adjustment to pH 3 (with glacial acetic acid) for column chromatography. Only in the case of the lauryl sulfate treated *S*-carboxymethylated protein did a significant precipitate appear after adjustment to pH 3; this amounted to *ca.* 5–10% of the total on a ninhydrin basis after alkaline hydrolysis (Hirs *et al.*, 1956). Column chromatography was conducted on Dowex 50-X2 (which had been sized into narrow ranges of particle sizes, according to the Moore *et al.* (1958) adaption of the hydraulic procedure of Hamilton (1958)); exponential gradients were employed with pyridine–acetic acid developers (Kimmel *et al.*, 1962), ranging from 0.2 to 2.0 M in pyridine and from pH 3.15 to *ca.* 9. Flow rates of 30 ml/hr through the column (at 50–55°) were used and either of two instruments had been employed (a Spinco 120 with a 2.2-mm cuvet and a Spinco 120B with a 6.6-mm cuvet); in addition, a stream splitter was utilized in one of the cases shown.

The procedure for peptide mapping of the tryptic digests was patterned after that of Katz *et al.* (1959) with paper electrophoresis (in a Savant LT-36 electrophoresis tank with the coolant initially at 10–12° conducted on Whatman 3MM filter paper (53 × 46.5 cm) in pyridine–acetic acid–H_2O (5:0.2:100 ml, pH 6.4, at 25°) and followed by descending chromatography in butanol–acetic acid–H_2O (4:1:5). Other details are given later on. Peptide areas were generally visualized with 0.1% ninhydrin in acetone (as a dipping reagent) followed by heating to 90–100° (the collidine–ninhydrin reagent of Canfield and Anfinsen (1963) was also employed in several cases as a spray). For detection of arginine-containing peptides, a modified Sakaguchi reagent (Jepson and Smith, 1953) was employed as a dipping reagent. To detect tryptophan-containing peptides, Ehrlich's reagent was used (Fincham, 1953) (but if the papers stood overnight, numerous yellow artifactual spots appeared, sometimes observable in areas containing no peptides, presumably derived from electrolytic degradation products of the buffers). Histidine-plus tyrosine-containing peptides were detected with the Pauly reagent (as given in Dawson *et al.*, 1959) and methionine-plus *S*-carboxymethylcysteine-contain-

[2] For the sake of brevity exact details on methods of these preparations have been omitted; however, they are available on request to S. A. K.

ing peptides with a platinic iodide dipping reagent (Toennies and Kolb, 1951). The isatin reagent (Acher *et al.*, 1950) for proline-containing peptides was successful for only a few proline-containing peptides, since with many of the other amino acid residues, varying degrees and shades of color were evident after periods of time.

Carboxyl-Terminal Group Studies. CARBOXYPEPTIDASE B DIGESTION. These were conducted with diisopropylphosphorofluoridate (DFP) treated carboxypeptidase B (Worthington lot 61A). Aliquots of carboxypeptidase B were dialyzed at 3° *vs.* deionized distilled H_2O (degassed) to remove traces of free amino acids and small peptides, clarified by centrifugation, and assayed spectrophotometrically (*ca.* 157 units/mg at 25°) with hippuryl-L-arginine (Mann) as substrate (Folk *et al.*, 1960). Crystals of preparation 5D of ATP–creatine transphosphorylase were dissolved in 0.001 M NH_4OH and dialyzed exhaustively *vs.* 0.01 M KCl, followed by distilled water, and a trace amount of insoluble material was removed by centrifugation. Protein concentrations were measured spectrophotometrically ($E_{280}^{1\%}$ 8.88 in 0.5 M phosphate (pH 7.0) for ATP–creatine transphosphorylase (Kuby *et al.*, 1962)) and an $E_{278}^{1\%}$ 21.4 at pH 7.6 (Folk *et al.*, 1960) was taken for the extinction coefficient of carboxypeptidase B.

Solutions of substrate protein were not frozen and were used directly.

Digestions from 0 to 6 hr were carried out at 1:40 mole ratios of carboxypeptidase B (mol wt 34,300; Folk *et al.*, 1960) to protein substrate, or approximately 1:100 on a weight basis, at 30° in 0.025 M borate (Na^+) (pH 9.02) with protein substrate concentrations at about 20 mg/ml. The methods were similar to those described earlier for carboxypeptidase A digestions (Olson and Kuby, 1964). Aliquots were removed at periodic time intervals into an equivalent volume of ice-cold 10% trichloroacetic acid; after removal of denatured protein by centrifugation in the cold, aliquots of the supernatant liquid were taken to dryness in glass tubes on a rotary evaporator, stoppered, and refrigerated until analyzed. The residues were dissolved in pH 2.2 sodium citrate buffer (Spackman *et al.*, 1958) and analyzed with the Spinco 120 B amino acid analyzer, employing a high-sensitivity cuvet (6.6-mm optical path) (Spinco Division, 1965) and accelerated flow conditions of 60 ml/hr. Controls were run under similar conditions to detect any amino acids released from carboxypeptidase B itself or from ATP–creatine transphosphorylase without protease; for the periods of the digestion, assays (with hippuryl-L-arginine) were conducted on the enzymatic activity of the carboxypeptidase B, in the presence of the substrate protein at 25°. The corrections for lysine released, as determined from the carboxypeptidase B controls, for any given time value, were never larger than 0.1 residue/mole of ATP–creatine transphosphorylase. Traces of amino acids other than lysine did not appear until after 60 min, but with values so small they could not be quantitated; only lysine appeared to be liberated (up to 5 hr) in significant amounts.

The carboxypeptidase B appeared to retain its activity through the digestion.

HYDRAZINOLYSIS. Aliquots (*ca.* 11 mg) of salt-free protein solution were pipetted into Pyrex tubes (9 × 1.5 cm o.d., with 10-cm stems of 1-cm o.d.), frozen, and taken to dryness by lyophilization (Mahowald *et al.*, 1962b). Samples of lysine and peptide controls were taken to dryness on a rotary evaporator. The dried residues were further dried in a vacuum (0.5 mm) oven at 74°, for 40 hr, with magnesium perchlorate as desiccant. Hydrazine (0.5 ml) (Eastman, White Label 95%+, or Matheson Coleman and Bell) was added to each bomb, frozen in Dry Ice–solvent, evacuated to 0.1 mm (oil pump), sealed, and placed at 85° (temperature-controlled, stirred silicone oil bath) for various periods of time. The contents were again frozen, and the bombs were opened and placed in desiccators containing H_2SO_4; by cautious evacuation to 0.5 mm (oil pump), the hydrazine was removed within 2–3 hr. Since quantitative recoveries of the free lysine (see below) were of more interest than quantitative removal of the hydrazides, the procedure followed that of Funatsu *et al.* (1964) but with the introduction of a back-extraction. The dried residues were dissolved and transferred quantitatively with glass-distilled water (total of 3 ml) to glass-stoppered tubes, 0.5 ml of isovaleraldehyde (Eastman, redistilled *in vacuo*) was added, and the mixture was shaken 2 hr at 25°. Following centrifugation in the cold for the protein samples (but unnecessary for lysine or peptide controls) to separate the organic phase, the isovaleraldehyde layer was back-extracted with two 1-ml water washes; the aqueous layers were combined, taken to dryness in glass tubes with a glass evaporator (bath temperature 30–40°), and kept refrigerated until analyzed. The residues were dissolved in the "diluting buffer" (pH 2.2) and analyzed with the Spinco 120 B equipped with the high-sensitivity cuvet (Spinco Division, 1965). Basic amino acids, in particular lysine, were separated either on 0.9 × 22 cm columns of Aminex Q 15-S resin (Bio-Rad) or on 0.9 × 55 cm of Aminex A-4 spherical resin (Bio-Rad). Longer columns than usual (Korenman *et al.*, 1966) were employed in order to detect the presence of any ornithine (which might be derived from arginine during hydrazinolysis); on the 22-cm column, ornithine appears as a shoulder on the ascending side of the lysine peak. Also, the 55-cm column proved necessary to completely resolve lysine from artifacts produced at the comparatively long period of hydrazinolysis (see also Braun and Schroeder, 1967). More care has to be taken in regeneration of these columns, since tightly bound hydrazides and their degradation products may be retained if the usual procedure as described is followed (Spinco Division, 1962); to check the thoroughness of the regeneration, standards were run after each analysis. The procedure was checked carefully and specifically for applicability only to lysine (for other C-terminal amino acids, the recent procedures of Braun and

Schroeder (1967) should be considered; compare also Locker, 1954). Akabori *et al.* (1956) had proposed a simple kinetic mechanism for hydrazinolysis of peptides (or proteins), *viz.*, a sequence of two first-order reactions (formation of the free C-terminal amino acid, followed by its exponential decay in hydrazine). Thus, it seemed likely that corrections for degradation of the carboxyl-terminal lysine (Olson and Kuby, 1964) could be made from measurements of the first-order degradation of free lysine in hydrazine (and as will be shown in the text). Also, by decreasing the temperature to 85°, higher yields of C-terminal lysine could be obtained than usually reported (*cf.* Niu and Fraenkel-Conrat, 1955), by minimizing its degradation. To test this procedure, a C-terminal lysine pentapeptide isolated from myokinase tryptic digestions and whose sequence was established as Ala-Asp-Thr-Ser-Lys (S. A. Kuby and L. J. Maland, unpublished data) was subjected to the same treatment at 105 and 85°. Yields were dramatically improved at 85°, and values of liberated lysine were measured at several time values, up to *ca.* 50 hr at 85°. Corrected yields of *ca.* 0.6–0.8 residue of lysine/mole, with uncorrected values of 0.5–0.6 mole, were obtained, and it appeared evident, by extrapolation, that at 70 hr corrected values of *ca.* 0.9 residue/mole could be obtained.

In the case of the protein, trace amounts of amino acids other than lysine could be detected chromatographically, and qualitatively they appeared to progressively increase after relatively long periods of hydrazinolysis (up to 70 hr); these included serine, threonine, glycine, alanine, leucine, and isoleucine, but in amounts less than 0.1 residue. Unavoidable hydrolysis of hydrazides are common occurrences (*e.g.*, Korenman *et al.*, 1966; Winstead and Wold, 1964; Locker, 1954; Spero *et al.*, 1965) and very likely these traces do not reflect any other C-terminal residues.

Results and Discussion

Physical Studies on the Native Enzyme

The earlier ultracentrifugation studies (Noda *et al.*, 1954) had been conducted before the advent of a suitable temperature control system (*viz.*, the Spinco RTIC unit) and only a few sedimentation velocity runs had been conducted, which were primarily for the purpose of establishing homogeneity. These data had not been extrapolated to zero protein concentration, and only a single diffusion run (kindly conducted by L. Gosting) was available at the time for estimation of the molecular weight by the classical equation of Svedberg (for sedimentation velocity and diffusion). Since a more quantitative estimation of the molecular weight of the native molecule was critical for the evaluation of the number- and weight-average molecular weights of dissociated products, it therefore necessitated a redetermination of its molecular weight. Moreover, the sedimentation velocity behavior, under several conditions where the enzyme is stable, was investigated over a large range of protein concentrations (which were permitted by the schlieren optics) to reveal, if any, the effects of interaction or dissociation (Nichol *et al.*, 1964).

Sedimentation Velocity. The sedimentation velocity behavior of the *native* molecule was explored, at 3° and at initial protein concentrations as high as 9–10 mg/ml, in the presence of 0.15 M KCl–0.01 M Tris–0.001 M EDTA (pH 7.9) or 0.15 M KCl–0.01 M phosphate (K^+)–0.001 M EDTA (pH 6.8). In addition, it was studied in the presence of 0.1 M β-mercaptoethanol at 3 (KCl–Tris–EDTA, pH 7.8) or 20° (KCl–phosphate, pH 6.8). The native enzyme, under all of these conditions, appeared to sediment as a single component (with symmetrical and monodisperse sedimenting boundaries), and with no evidence of aggregation[3] or dissociation.

The observed sedimentation coefficients (at 3° and buffer) decrease slightly with an increase in protein concentration over the range of protein concentrations explored (Figure 1). However, in the presence of 0.15 M KCl-0 01 M phosphate–0.001 M EDTA (pH 6.8) the dependency of $s_{3°,b}$ *vs.* $\bar{c}$ is best described by a two-term expression to account for the small departure from linearity at relatively high protein concentrations (where $\bar{c}$ is expressed in milligrams per milliliter of the average boundary concentration), *viz.*, $s'_{3°,b} = (3.32 - 0.0005\bar{c} - 0.0022\bar{c}^2) \times 10^{-13}$ sec, and which is indicative, perhaps of a weakly interacting system (Nichol *et al.*, 1964). Similarly, in 0.15 M KCl–0.01 M Tris–0.001 M EDTA (pH 7.8) although there appears to be some curvature in the $s'_{3°,b}$ *vs.* $\bar{c}$ plot at high values of $\bar{c}$, the extent of curvature is somewhat less than in the former case and is best described by $s'_{3,b} = (3.30 - 0.0065\bar{c} - 0.0015\bar{c}^2) \times 10^{-13}$ sec. The extrapolated values (facilitated by these two-term expressions) at $\bar{c} \rightarrow 0$, are identical within experimental error, after correction to the reference states of 20° and water, and correspond to $s^0_{20°,w}$ values of 5.31 and 5.27 S, respectively.

Free Diffusion and Molecular Weight by Sedimentation Velocity and Diffusion ($M_{s,D}$). Diffusion coefficients (Figure 2), as a function of the average boundary concentrations, were measured under one set of conditions (0.15 M KCl–0.01 M Tris–0.001 M EDTA, pH 7.8) and again required the use of a two-term expression to account for all the data, *viz.*, $D'_{3°,b} = (3.58 - 0.023\bar{J} + 0.0006\bar{J}^2) \times 10^{-7}$ cm²/sec (where the average boundary concentration has been expressed in terms

[3] It is of interest that in the case of a three-times-crystallized sample (preparation 3D, which had originally shown identical sedimentation behavior to the above) which was then lyophilized in 0.01 M glycine (pH 9), followed by storage at −10° for 2 years, in addition to the native enzyme, a trace of a heavier sedimenting component could be observed with an $s_{20,w}$ of *ca.* 6.8 S (at 9.4-mg/ml initial protein concentration). This trace of a heavier component disappeared after addition of 0.1 M mercaptoethanol (in 0.15 M KCl–0.01 M phosphate, pH 6.8). Thus, interdisulfide formation very likely can take place to a small extent in lyophilized samples, after prolonged periods of storage.

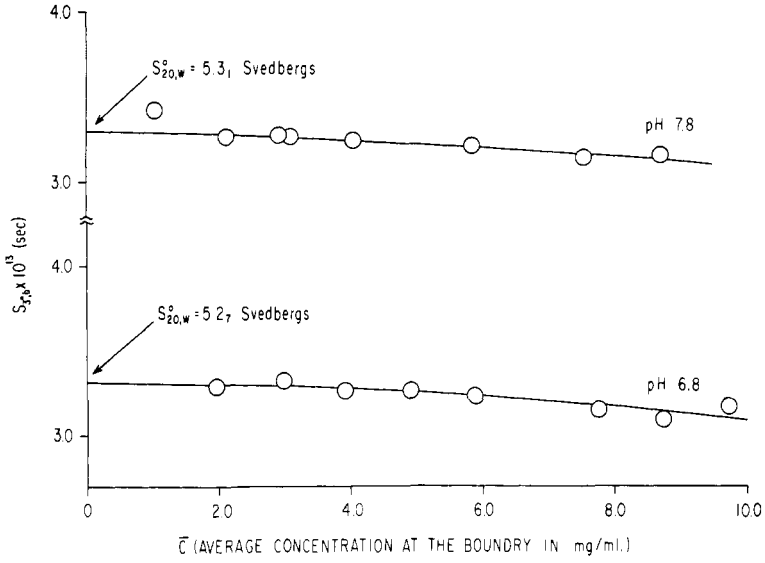


FIGURE 1: Sedimentation coefficients of ATP–creatine transphosphorylase as a function of protein concentration at 3° and at pH 7.8 and 6.8. Concentration at the boundary ($\bar{c}$) in milligrams per milliliter is the average value between the beginning and the end of the experiment, as determined by correcting for the radial dilution of the sector-shaped cell. Upper curve: in 0.15 M KCl–0.01 M Tris–0.001 M EDTA (pH 7.8). $s'_{3^\circ,b} = (3.30 - 0.0065\bar{c} - 0.0015\bar{c}^2) \times 10^{-13}$ sec. Lower curve: in 0.15 M KCl–0.01 M phosphate–0.001 M EDTA (pH 6.8). $s'_{3^\circ,b} = (3.32 - 0.0005\bar{c} - 0.0022\bar{c}^2) \times 10^{-13}$ sec.

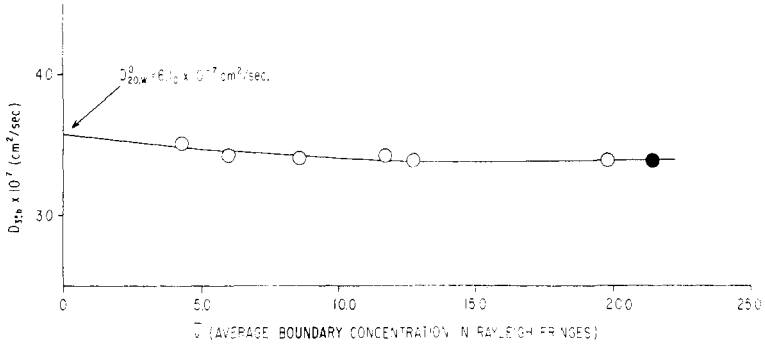


FIGURE 2: Diffusion coefficients of ATP–creatine transphosphorylase as a function of protein concentration, at 3.00°, pH 7.8, 0.15 M KCl–0.01 M Tris–0.001 M EDTA. Data were obtained in both the 11- and 2-ml modified Tiselius cells. The concentrations have been expressed in terms of average number of Rayleigh fringes ($\bar{J}$) at the protein boundary (*i.e.*, $J_0/2$) corrected for differences in optical paths of the two cells and referred to that of the 2-ml cell. The initial protein concentrations ranged from about 2.2 mg/ml to about 9.86 mg/ml over the entire plot. The one value indicated by ● was determined by Gosting (as quoted by Noda *et al.* (1954)) using Gouy interferometric optics. The two-term expression for the entire plot is $D'_{3^\circ,b} = (3.58 - 0.023\bar{J} - 0.00069\bar{J}^2) \times 10^{-7}$ cm²/sec.

of Rayleigh fringes, $\bar{J}$). The last point in the plot of Figure 2 is that of L. Gosting (given in Noda *et al.*, 1954), obtained at pH 9.0, 0.1 ($\Gamma/2$) glycine, 25°, and corrected to the temperature and viscosity of these measurements). As may be seen, it shows an excellent fit to the over-all plot. Extrapolation of the diffusion data to $\bar{J} = 0$, after correction to 20° and water, yields a value of $D^0_{20^\circ,w} = 6.10 \times 10^{-7}$ cm²/sec. Application of the classical Svedberg equation for sedimentation velocity and diffusion, with the use of the values for $D^0_{3^\circ,b}$ and $s^0_{3^\circ,b}$ to eliminate any inherent errors in the Stokes–Einstein relation (Gosting and Morris, 1949) and $\bar{V}^0_{app,3^\circ}$ (extrapolated from the data of Noda *et al.*, 1954), gives a calculated value for the apparent weight-average molecular weight ($M_{s,D}$) of 82,700 (with an estimated uncertainty of about 1600). This value may be compared with the earlier value of 81,000 (Noda *et al.*, 1954) which had been estimated from single values of the sedimentation velocity coefficient and for the diffusion coefficient

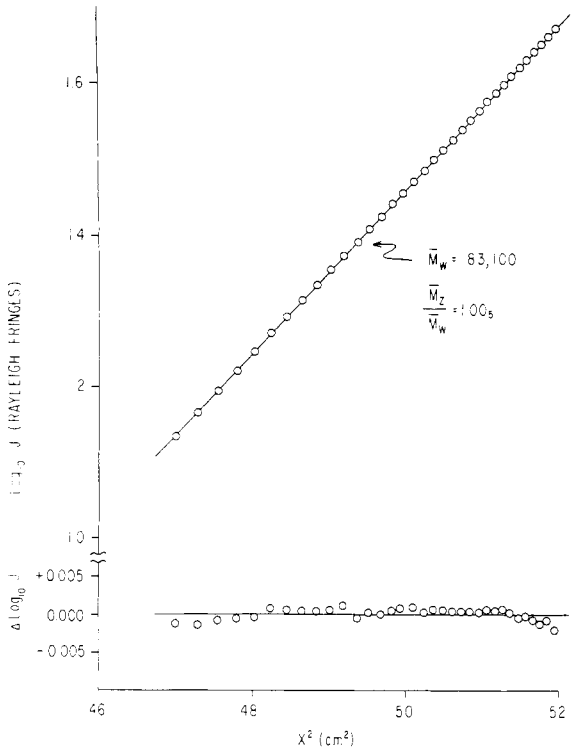


FIGURE 3: A molecular weight determination of ATP–creatine transphosphorylase from a sedimentation equilibrium experiment, expressed in terms of a plot of log J (log of the protein concentration in Rayleigh fringes) *vs.* x^2 (square of the distance, in centimeters squared, from the axis of rotation). The initial concentration of protein was 6.6 mg/ml, in 0.15 M KCl–0.01 M Tris–0.001 M EDTA (pH 7.8) at 3.0°. The protein was sedimented initially at 11,250 rpm for 4.5 hr and then the speed was reduced to 6995 rpm. The time for the data given here was 92.5 hr after the equilibrium speed (6995 rpm) was reached at 3.0°, and no significant differences were noticed between 65 and 92.5 hr. For the lower "deviation" plot $\Delta(\log J)$ is the difference, at a given value of x^2, between the least-squares straight line and the experimental points.

at *ca.* 9-mg/ml initial protein concentration. Compensation of the small concentration effects in both sedimentation and diffusion led to the fortuitously close agreement.

Coincident with these studies, which had employed Rayleigh interferometric optics, a value for the refractive index increment could be assigned, for 3°,b (0.01 M Tris–0.15 M KCl–0.001 M EDTA, pH 7.8), *viz.* $(\Delta n/\Delta c) = 1.84 \times 10^{-3}$ (g/100 ml)$^{-1}$ for 546 mμ. This value will likely prove useful in later studies on the possible effects of denaturants on the refractive index increments, extinction coefficients, and in particular partial specific volume of this protein (for a discussion on this latter point, *cf.* Noelken, 1967; Schachman and Edelstein, 1966; Marler *et al.*, 1964; Linderstrøm-Lang, 1950; Kielley and Harrington, 1960; Reithel and Sakura, 1963; Reithel *et al.*, 1964) which may be of general interest from a theoretical standpoint.

TABLE I: Molecular Weight Determinations of ATP–Creatine Transphosphorylase by Sedimentation Equilibrium at 3.0° and pH 7.8, 0.15 M KCl–0.01 M Tris–0.001 M EDTA, at Several Initial Protein Concentrations (Expressed in Rayleigh Fringes).

Initial Concn of Protein (Rayleigh fringes)	M_{equil} (or $\bar{M}_w$)
26.65	83,100
21.72	82,000
18.99	83,300
15.42	83,800
11.23	80,900
	82,600 ± 2,300 (2 std dev)

Sedimentation, Equilibrium, and Molecular Weight ($\bar{M}_w$). Confirmation of the above value for the molecular weight was made by true sedimentation equilibrium analyses (Richards and Schachman, 1959) with a typical analysis presented (Figure 3) in a plot of log J *vs.* X^2 at sedimentation equilibrium. The lower fringe deviation graph (Richards and Schachman, 1959) illustrates the satisfactory internal precision of the interferometric data, and only near the extreme ends of the liquid column do significant errors in measurement become manifest. The ratio of the z-average to the weight-average molecular weight for this run is close to the theoretical value of 1.0 (1.005 for Figure 3) attesting to the homogeneity of the preparation and to the lack of dissociation under these conditions.

In Table I are summarized the apparent weight-average molecular weights ($\bar{M}_w$) estimated by sedimentation equilibrium for several initial protein concentrations. Since there is no *significant* trend over the relatively narrow protein concentration range which could be conveniently explored by this technique, a mean value of 82,600 ± 2300 (*i.e.*, ±2 std dev) may be calculated, which is in excellent agreement with the above value of $M_{s,D}$. The less accurate procedure of high-speed sedimentation equilibrium (Yphantis, 1964) at an initial protein concentration far below the lowest concentration given in Table I (*i.e.*, *ca.* 0.2 mg/ml) has yielded a value which is not in serious disagreement with this value, but with a larger uncertainty (see below). A value of 82,600 is then taken as the most reliable estimate of the molecular weight of the native enzyme; there would appear to be no evidence for dilution effects, *i.e.*, dissociation at high dilutions.

However, slight nonideality effects may be expected at relatively high protein concentrations (higher than

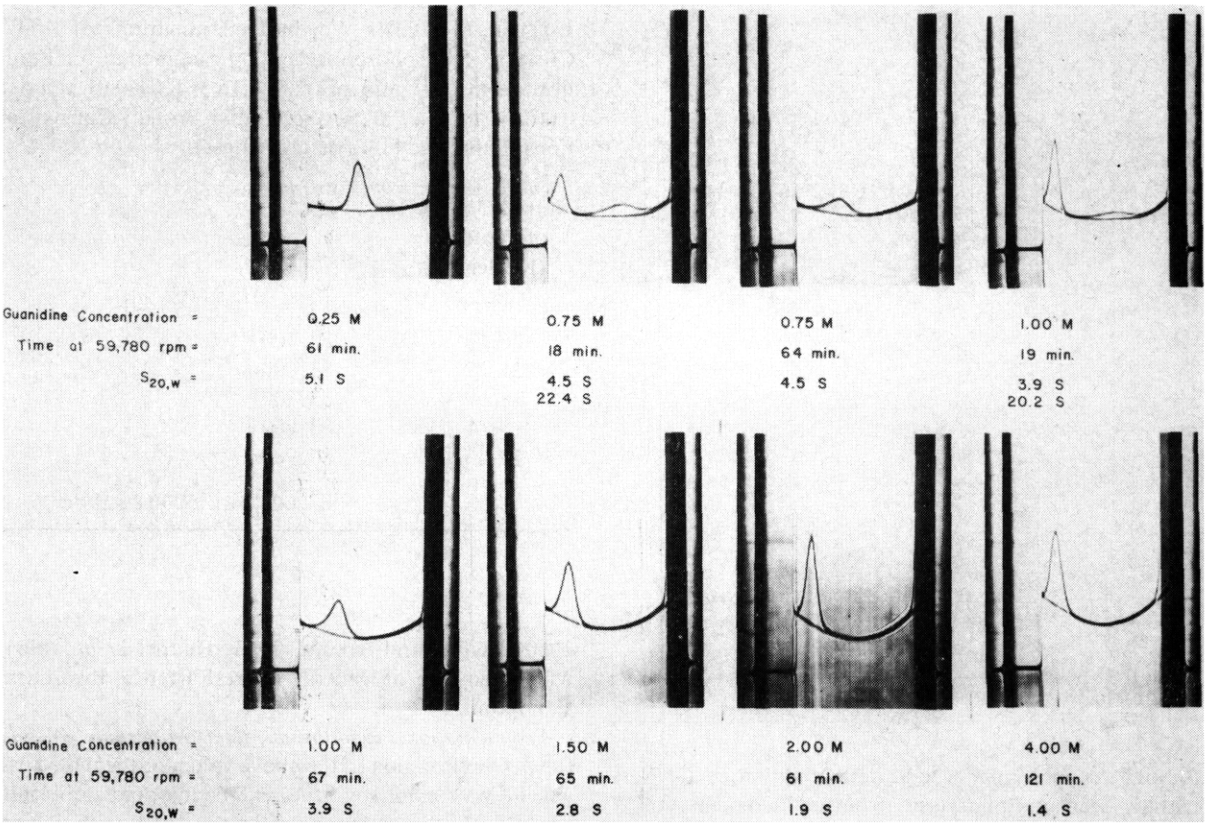


FIGURE 4: Effect of guanidine hydrochloride (in 0.1 M β-mercaptoethanol–0.001 M EDTA, pH 6.3) on the sedimentation velocity behavior of ATP–creatine transphosphorylase at 20°, with an initial protein concentration of 8.0 mg/ml. The schlieren photographs reproduced above were taken at the time indicated after the centrifuge had reached a speed of 59,780 rpm. Menisci are on the left. The schlieren diaphragm (phase plate) angle was set at 70°.

those explored in Table I) as revealed by the sedimentation velocity and translational diffusion studies (*vide supra*).

Physical Studies on the Polypeptide Chains in Several Dispersing Agents

Sedimentation Velocity Studies. In the presence of increasing concentrations of guanidine hydrochloride (0.1 M β-mercaptoethanol–0.001 M EDTA, pH 6.3, at 20°) and above 0.25 M, a progressive and marked change in the sedimentation velocity behavior of the native enzyme occurred (Figure 4) at an initial protein concentration of *ca.* 8 mg/ml. With increasing concentrations of guanidinium chloride, a progressive decrease in the sedimentation coefficient, corrected to 20° and water, occurred. At 0.25 M (or below) and again above 2.00 M guanidine, only a single sedimenting species was evident; between these concentrations, however, the system showed a tendency toward aggregate formation (with species sedimenting in the neighborhood of 20 S). With an increase in guanidinium chloride concentration, the concentration of the aggregates progressively declined, until at 2.00 M, they had disappeared entirely. When the $s_{20,w}$ values of the lighter (and nonaggregated species) are plotted *vs.* the molarity of the guanidine concentration (Figure 5), a sigmoidal curve results; the data show that the reagent causes a marked and progressive decrease in the sedimentation coefficient, from initially a value of *ca.* 5.2 S for the native enzyme at this initial protein concentration to a limiting value of *ca.* 1.4 S, which is approached asymptotically at high guanidine (*i.e.*, 4 M) concentrations. Molecular weights measured under these conditions of 4 M guanidinium chloride (see below) reveal that complete dissociation into subunits had occurred. Moreover, dissociation at high guanidinium chloride concentration was apparently accompanied by a transition from the native compact and globular macromolecular structure, with a calculated frictional ratio (f/f_0) of only 1.21 and an axial ratio of 4.4 for an assumed anhydrous prolate ellipsoid (Perrin, 1936), to a structure which had likely undergone appreciable unfolding and other conformational changes as revealed by an $f/f_0 = 2.92$ (calculated with an $\bar{M}_w = 41{,}300$ (see below), $s_{20,w} = 1.44$ S, and $\bar{V}_{20°} = 0.735$) which is typical of random coils.

A single run at 20° was conducted at a high NaCl concentration (1.4 M NaCl–0.001 M EDTA–0.1 M mercaptoethanol, pH 6.3) as a comparison for the run made in the presence of 1.5 M guanidinium chlo-

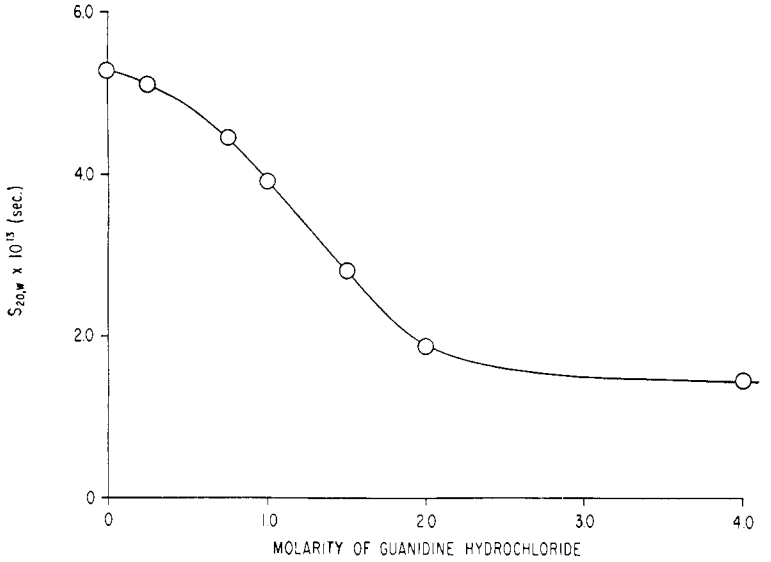


FIGURE 5: Effect of guanidine hydrochloride (in 0.1 M β-mercaptoethanol–0.001 M EDTA, pH 6.3) on the sedimentation coefficient ($s_{20,w}$) of the nonaggregated species of ATP–creatine transphosphorylase.

ride. Only a single symmetrical boundary of 4.7 S was observed in 1.4 M NaCl, in contrast to the species sedimenting with a value of 2.8 S in 1.5 M guanidinium chloride. The difference between an $s_{20°,w}$ value of 4.7 and 5.2 S (for the 0.15 M KCl systems) at *ca.* 8 mg/ml may be only slight, considering the uncertainties involved in correcting to the reference state of water at 20°.[4] Thus, NaCl is rather ineffective as a disruptive agent compared to guanidine at similar concentrations.

Compared to the guanidine systems, urea shows some qualitative differences at intermediate concentrations (see Figure 6). In aqueous 4 M urea solutions, containing 0.15 M KCl–0.01 M phosphate (K^+)–0.1 M β-mercaptoethanol (pH 6.8) after 13 hr at 20° (initially, *ca.* 8.3-mg/ml total protein concentration), interestingly, there is a disappearance of material from the boundary which might correspond to the native molecule (with an $s_{20,w} \cong 4.7$ S) and an accompanying formation of a very slowly sedimenting species (of *ca.* 2.1 S); in addition a small amount of aggregated material (of *ca.* 29 S) is evident. At 3° (and after 22 hr) the heavy aggregate material is absent, but once again in 4 M urea and under otherwise identical conditions (including 0.1 M β-mercaptoethanol), there is still an apparent resolution of the pattern into two components, one of *ca.* 5.1 S and the other of *ca.* 1.9 S, which presumably correspond to the native and dissociated species, respectively. In 8 M urea (Figure 6), only a single sedimenting species is observed, in the presence or absence of mercaptoethanol, at 20°; under these conditions, a dissociated species has resulted (see below for its molecular weight) of *ca.* 1.8 S, a value not too dissimilar from the limiting value obtained in 4 M guanidinium chloride (if one considers the errors involved in correction to 20° and water). Dissociation did not require the presence of mercaptoethanol, but it appears to have enhanced the stability of the system.

Interestingly, there is a slight time dependency (in addition to the effect of temperature) in the case of 4 M urea, as well as specific effects due to pH stability (*cf.* Simpson and Kauzmann, 1953; Westhead, 1964). At 3° and where the sedimentation velocity run was made immediately after adjustment of the native enzyme (in solution) to 4 M urea (and 0.15 M KCl–0.01 M Tris (pH 7.8)–0.1 M mercaptoethanol) only a single component corresponding to the native enzyme is obtained ($s_{20°,w} = 4.9$ S). However, the same solution after 24 hr at 3° displayed in addition to the 4.9S component only a trace amount of 2.7S component. Apparently, the protein in the Tris–urea (pH 7.8) system is far more stable than in the phosphate–urea (pH 6.8) system (*cf.* Figure 6) (but note that the latter system was measured after dissolving lyophilized material in the 4 M urea solution, whereas the former system was prepared by dilution of an enzyme solution with 6 M urea to yield a final 4 M solution).

Lauryl sulfate proved to be unique among the disruptive agents explored. Results with the double-sector cell displayed an interesting phenomenon. With increasing concentrations of lauryl sulfate (from 0.0087 to 0.035 M), there was a concomitant increase apparently in micelle formation of the lauryl sulfate itself, in the solvent sector of the cell (see Figure 7)

[4] The diffusion coefficient for the run made in 1.4 M NaCl at *ca.* 8-mg/ml initial protein concentration was approximated by boundary-spreading analysis (Svedberg and Pedersen, 1940; Baldwin, 1957) to give a $D_{20,w} \cong 5.6 \times 10^{-7}$ cm²/sec. Use of an $s_{20,w} = 4.7 \times 10^{-13}$ S, and $\bar{V}_{20°} = 0.744$, yields a value for $M_{s,D} \cong 80{,}000$, and therefore it does not appear that any significant dissociation takes place at high salt concentration.

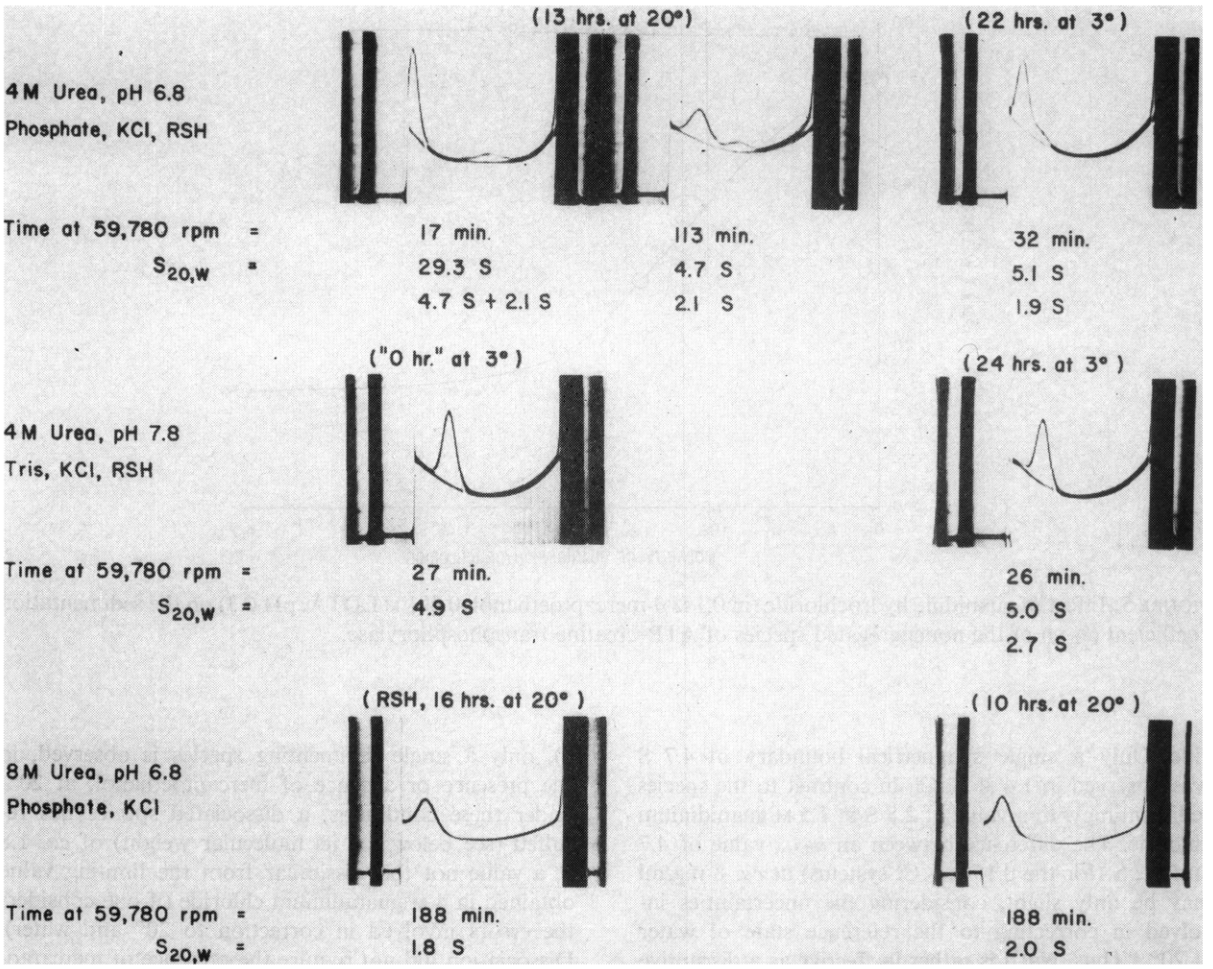
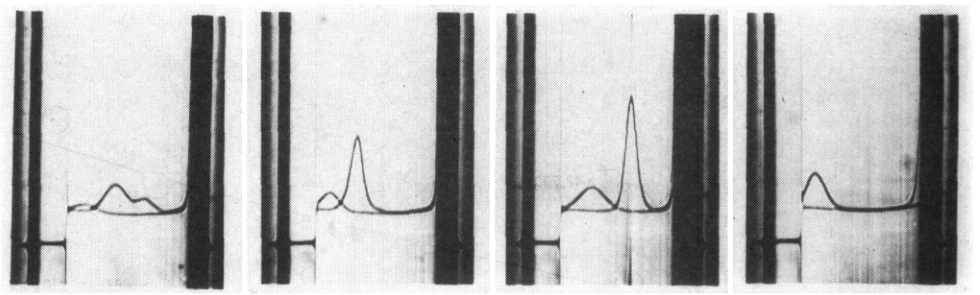


FIGURE 6: Effect of urea on the sedimentation velocity behavior of ATP–creatine transphosphorylase with an initial protein concentration of 8 mg/ml. The schlieren photographs reproduced above were taken at the time indicated after the centrifuge had reached a speed of 59,780 rpm. Menisci are on the left. The schlieren diaphragm (phase plate) angle was set at 70°.

with a calculated $s_{20,w}$ of *ca.* 1.1 S for the micelle. This was confirmed with "blank" runs in the absence of protein (see last frame of Figure 7; compare also Sabato and Kaplan, 1964; Granath, 1953). However, in the solution sector containing the protein, no evidence of micelle formation could be observed. It was as if the protein had bound almost quantitatively the lauryl sulfate added to the solution and therefore prevented or eliminated the formation of the micelles. (See Putnam, 1948, for an older but still pertinent discussion of lauryl sulfate micelle formation and interactions with proteins.) These results may be compared with those of Edelhoch and Lippoldt (1960) on thyroglobulin, where by sedimentation and light-scattering studies they inferred a rather avid binding of lauryl sulfate to this protein, and had concluded that at low concentrations of detergent, dissociation occurred, but with a minimum extent of unfolding.

In the present case, preferential binding makes interpretation difficult, but nevertheless, at 8-mg/ml initial protein concentration, and in the presence of 0.0087 M lauryl sulfate (0.15 M NaCl–0.1 M mercaptoethanol–0.001 M EDTA, pH 6.3, and 20°), the resulting sedimentation pattern is similar to that obtained in 4 M urea (0.01 M phosphate–0.15 M KCl, 22 hr at 3°). Two species appear (5.2 and 3.0 S) (*cf.* Figure 7 with Figure 6). The former value corresponds remarkably well to the value obtained for the native enzyme, corrected to 20° and water (at that initial concentration), and the latter may be compared with the following calculated values of $s_{20,w}$: (a) of 4.2 S, for a dissociated species, essentially spherical in shape and with an $\bar{M}_w$ = 41,300 and $\bar{V}$ = 0.735; and (b) of 3.5 S, for a dissociated polypeptide chain of $\bar{M}_w$ = 41,300 which had retained the same frictional ratio (f/f_0 = 1.21) as the native molecule (and the same calculated axial ratio of 4.4 for an assumed anhydrous prolate). A value of 3.5 S then would correspond to a hypothetical and ordered dissociated half-molecule with identical f/f_0 as the native molecule.



Lauryl Sulfate Concentration =	0.0087 M	0.017 M	0.035 M	0.035 M (Without Protein)
Time at 59,780 rpm =	95 min.	92 min.	182 min.	88 min.
$S_{20,w}$ in Solution Sector =	5.2 S 3.0 S	3.4 S	2.5 S	
$S_{20,w}$ in Solvent Sector =	1.2 S	1.3 S	1.1 S	1.1 S

FIGURE 7: Effect of lauryl sulfate in 0.15 M NaCl–0.1 M β-mercaptoethanol–0.001 M EDTA (pH 6.3) on the sedimentation velocity behavior of ATP–creatine transphosphorylase with an initial protein concentration of 8.0 mg/ml. The schlieren photographs reproduced above were taken at the time indicated after the centrifuge reached a speed of 59,780 rpm. Menisci are on the left. The schlieren diaphragm (phase plate) angle was set at 70°. The solvent sector component was identified by blank runs, shown on the extreme right.

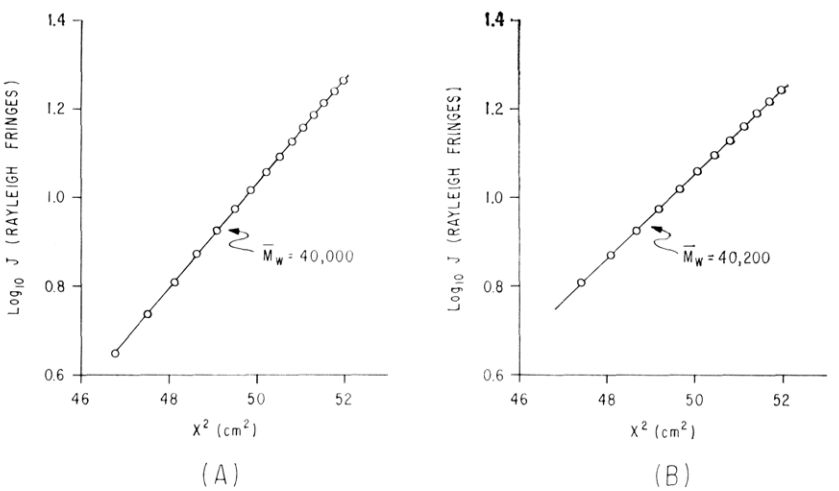


FIGURE 8: Molecular weight determinations of ATP–creatine transphosphorylase in 4 M guanidine solutions from sedimentation equilibrium experiments, expressed in terms of plots of log J (log of the protein concentration in Rayleigh fringes) *vs.* x^2 (square of the distance, in centimeters squared, from the axis of rotation). The initial concentration of the protein was 2.5 mg/ml. (A) With 0.1 M β-mercaptoethanol (pH 6.3) at 20.0°. The protein was sedimented initially at 25,980 rpm for 3 hr and the speed was reduced to 12,590 rpm. The time for the data given here was 72 hr after the equilibrium speed (12,590 rpm) was reached at 20.0°, and no significant differences were noticed between 22 and 72 hr. (B) With 0.001 M EDTA, but *without* β-mercaptoethanol (pH 6.3), at 20.0°. The protein was sedimented initially at 23,150 rpm for 4 hr and the speed was reduced to 11,250 rpm. The time for the data given here was 21 hr after the equilibrium speed (11,250 rpm) was reached at 20.0°. Slight differences were noticed between 21 and 46 hr. See text.

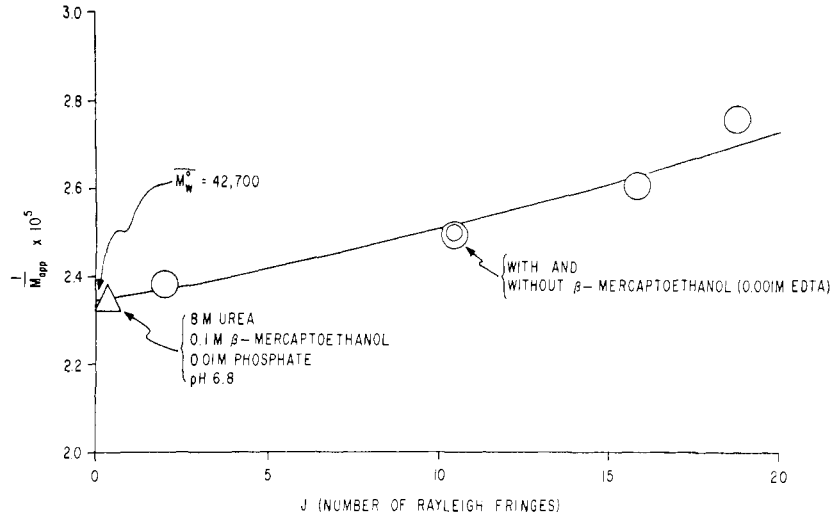


FIGURE 9: Apparent weight-average molecular weights of ATP–creatine transphosphorylase in 4 M guanidine hydrochloride–0.1 M β-mercaptoethanol (pH 6.3) at 20°, as a function of protein concentration. The two-term equation, corresponding to the line drawn, is $1/M_{app} = (2.34 + 0.0133J + 0.00031J^2) \times 10^{-5}$.

It is clear that at 0.25% lauryl sulfate (or 0.0087 M) both the undissociated molecule, and possibly the dissociated product as well,[5] might have retained a significant degree of ordered structure; this conclusion, although subject to the restrictions involved in such tenuous calculation, is, nevertheless, in agreement with the conclusions that Edelhoch and Lippoldt (1960) had reached in the case of another protein. Increasing the lauryl sulfate concentration to 0.017 M yields an $s_{20,w}$ value of actually 3.4 (Figure 7) (extraordinarily close to the calculated value for the hypothetical dissociated and ordered polypeptide moiety), with only a single sedimenting boundary evident in the solution sector. Upon doubling the lauryl sulfate concentrations, the Svedberg coefficient decreases to about 2.5 S, and this further change may be the final result of the preferential binding of lauryl sulfate, and/or a change in the frictional coefficient (*cf.* Sabato and Kaplan, 1964).

Studies at very acid or at very alkaline pH values were not considered worthwhile to explore for the present. The effects of charge, nonidealty, as well as concomitant aggregation problems all combine to make such studies difficult to decipher and interpret (*cf.*, *e.g.*, studies on hexokinase by Derechin *et al.* (1966), but see Dawson *et al.* (1967)).

Sedimentation Equilibrium Studies. The sedimentation velocity studies contained information for conditions required for complete dissociation, but it was of course necessary to evaluate the molecular weight distributions of the completely dissociated products. Decreases in the sedimentation coefficients, by themselves, could be attributed to dissociation of macromolecules into compact subunits, or to an increase in the frictional coefficient resulting from swelling or elongation of the molecule without dissociation, or to combinations of dissociation and disorganization.

Under conditions of high guanidinium concentrations, where drastic alterations in the hydrodynamic behavior had occurred (see Figures 4 and 5) and where it was presumed to have resulted in complete dissociation, true sedimentation equilibrium runs indeed revealed that the weight-average molecular weight of the system had decreased to approximately one-half (Figure 8), *viz.*, to *ca.* 40,000, at *ca.* 2.5-mg/ml initial protein concentration. Also, it was evident that the value of $\overline{M}_w$ did not change in the presence or absence of β-mercaptoethanol, at pH 6.3. Thus, interchain disulfide formation can be eliminated as a bridge between the polypeptide chains[6] and the native mole-

[5] An approximate estimation of the diffusion coefficients by boundary-spreading analysis (Svedberg and Pedersen, 1940; Baldwin, 1957) was made for the two sedimenting species shown in Figure 7 for 0.0087 M lauryl sulfate and 8-mg/ml initial total protein concentration. Application of the Svedberg equation together with their measured sedimentation coefficients yield estimated values for the molecular weights of these species of, very approximately, 35,000 and 80,000, respectively, in confirmation of the conclusions reached above, but beyond 0.0087 M, the lauryl sulfate binding corrections to the molecular weight (Hersch and Schachman, 1958) become too large to permit reasonable approximations of $M_{s,D}$ by this method.

[6] Although mercaptoethanol by itself does not cause dissociation and is not required for splitting of the molecules into the polypeptide chains (compare also the sedimentation velocity studies, which show no significant differences with or without the addition of β-mercaptoethanol), nevertheless, its addition improves the stability of the system. In the case of the equilibrium sedimentation run described above, in 4 M guanidinium chloride, equilibrium appeared to be reached in *ca.* 25 hr after acceleration, for these conditions, but slight aggregation became evident at the bottom of the cell if the time were extended much beyond

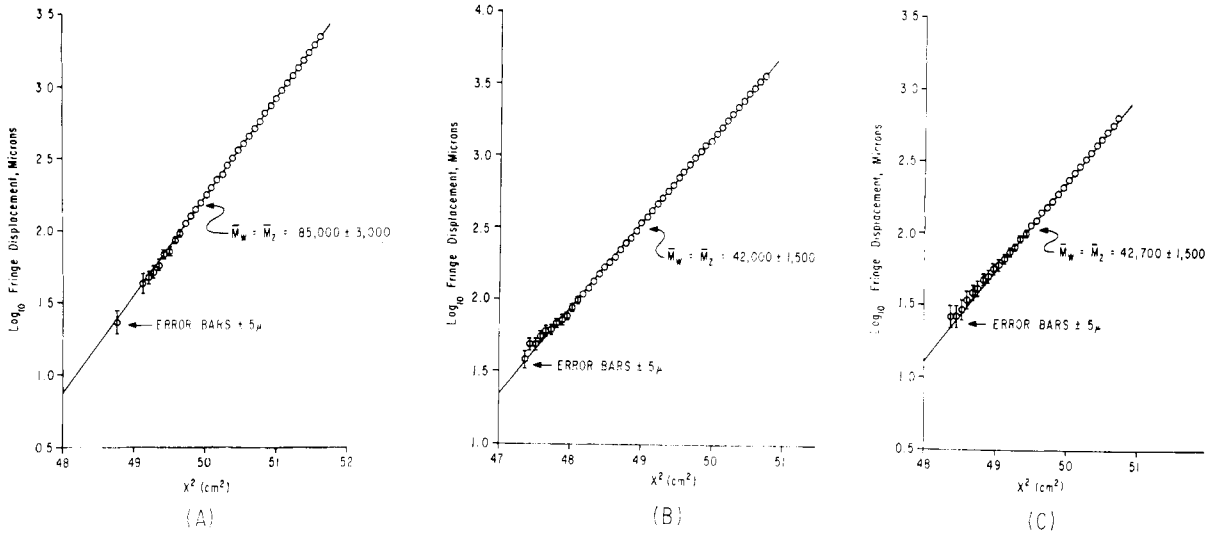


FIGURE 10: Molecular weight determinations of ATP–creatine transphosphorylase by the sedimentation equilibrium procedure, according to Yphantis (1964), expressed in terms of logarithm of the fringe displacement in microns (blank corrected) *vs.* x^2 (square of the distance, in centimeters squared, from the axis of rotation). (A) Protein (0.2 mg/ml) in 0.15 M KCl–0.01 M Tris–0.001 M EDTA–0.1 M β-mercaptoethanol (pH 7.8) at 3.0°. The time for the data given here was 28 hr at 17,250 rpm. (B) Protein (0.6 mg/ml) in 4 M guanidine hydrochloride–0.001 M EDTA–0.1 M β-mercaptoethanol (pH 6.3) at 20.0°. The time for the data given here was 52 hr at 27,690 rpm. (C) Protein (0.1 mg/ml) in 8 M urea–0.001 M EDTA–0.01 M phosphate (K^+)–0.1 M β-mercaptoethanol (pH 6.3) at 20.0°. The time for the data given here was 52 hr at 29,500 rpm. The indicated ranges in the plots correspond to ±5-μ fringe displacement; only points with a net displacement greater than 100 μ have been used for calculations (see Yphantis, 1964).

cule would appear to consist of two polypeptide chains. At this initial concentration, the linear plots of log J *vs.* X^2 and the fact that $\bar{M}_w \cong \bar{M}_z$ (and, see below, by the Yphantis (1964) technique $\bar{M}_n \cong \bar{M}_w \cong \bar{M}_z$) would indicate that the two constituent polypeptide chains per native molecule are essentially alike in their molecular weights, within the precision of the methods.

At higher initial protein concentrations (*ca.* 4.5 mg/ml) a slight concave downward curvature in the log J *vs.* X^2 plots was evident which is indicative of thermodynamic nonideal effects (Schachman, 1959) or preferential binding in the guanidinium system, with a subsequent decrease in the measured weight-average molecular weight throughout the entire contents of the cell (*i.e.*, $\bar{M}_w$ decreased to *ca.* 38,000). Therefore, to eliminate some of the nonideal effects, equilibrium measurements were conducted as a function of initial protein concentrations (Figure 9). Over the range in initial concentrations explored (where the lowest values were estimated by the high-speed Yphantis (1964) procedure), the data could be fitted to the expression $1/M_{app} = (2.34 + 0.0133J + 0.00031J^2) \times 10^{-5}$ (where M_{app} is the measured value of $\bar{M}_w$, for a particular initial protein concentration expressed in Rayleigh fringes, J), which provided a suitable extrapolation to zero protein concentration, *viz.*, $\bar{M}_w^0 = 42{,}700 \pm 1300$ (for two standard deviations). The data for 8 M urea at the lowest protein concentration (*ca.* 0.1 mg/ml, $M_{app} \cong 42{,}700$) were also fitted to the calculated curve. As pointed out by Schachman and Edelstein (1966) [and by Reithel *et al.* (1964)], extrapolation to zero protein concentration might not be expected to eliminate all the effects of preferential binding of solvent components (including urea or guanidine); nevertheless, the agreement that the urea data (at low protein concentrations) showed with the guanidine data (extrapolated to zero protein concentration) would indicate that most, if not all, of the preferential binding effects disappeared at low protein concentrations. It is likely that the greatest uncertainty lies in the selection of $\bar{V}$ (*cf.* Kawahara and Tanford (1966) and Schachman and Edelstein (1966); also see Noelken (1967), Marler *et al.* (1964), Kielley and Harrington (1960), Reithel *et al.* (1964), Linderstrøm-Lang (1950)) and, in this case, the value selected for the dissociated system was that calculated from the amino acid composition (Noltmann *et al.*, 1962), *viz.*, 0.735 cc/g (see also Experimental Section). An uncertainty of ±0.01 cc/g in $\bar{V}$ would mean that the difference between, *e.g.*, 41,300 for a hypothetical half-molecule

this point, and $\bar{M}_w$ had increased to *ca.* 43,200 in 50 hr. In contrast to this case, where β-mercaptoethanol was introduced, equilibrium runs as long as 75 hr could be made with no evidence of aggregation and no trend in $\bar{M}_w$ from 25 to 75 hr. Thus, it would appear that aggregation *via* interchain disulfide formation might possibly take place under some conditions (see also footnote 3).

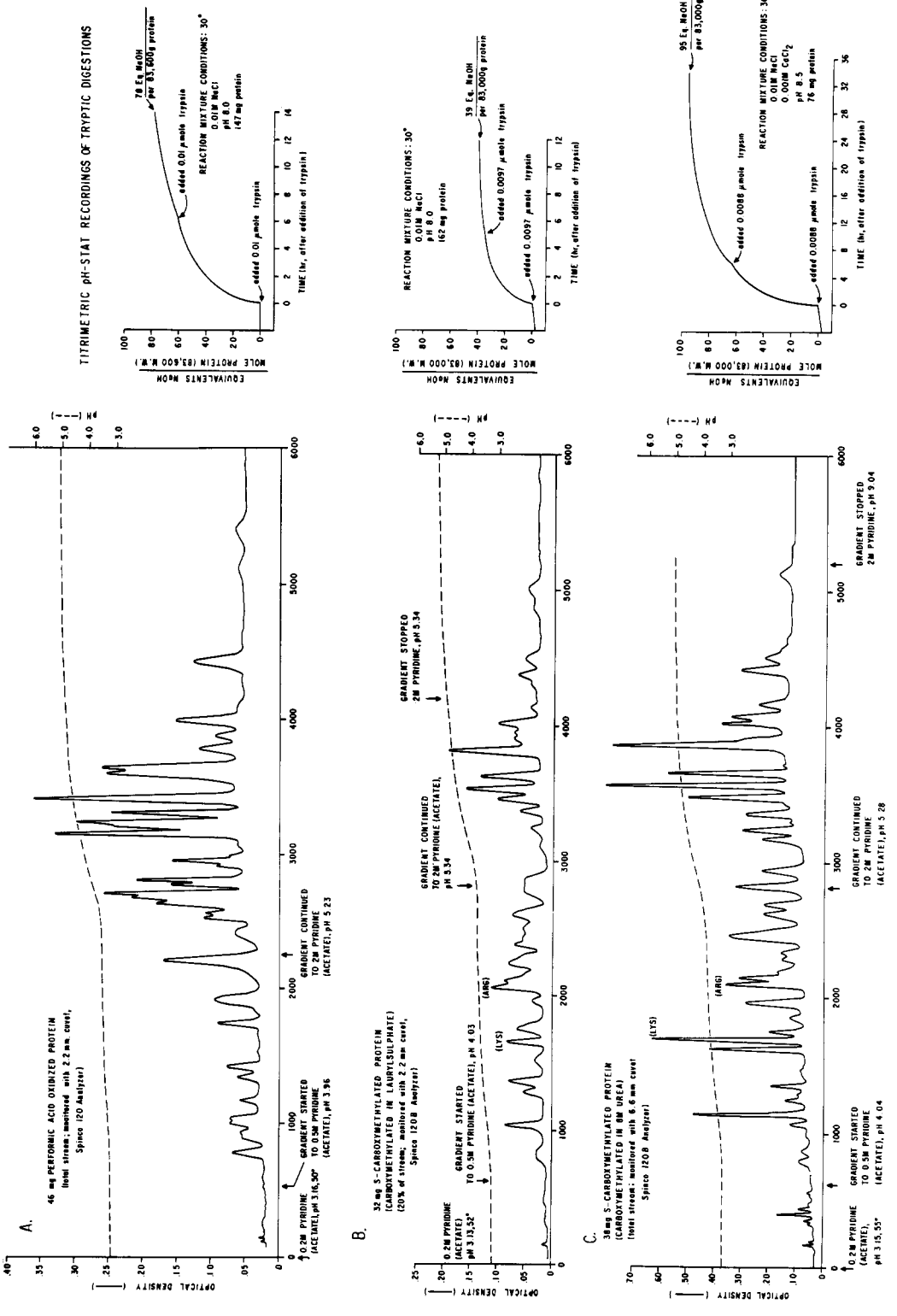


FIGURE 11: Titrimetric pH-Stat recordings of tryptic digestions and chromatography of the tryptic digests of ATP–creatine transphosphorylase derivatives (on Dowex 50-X2, 0.9 × 150 cm, 30 ml/hr). Descriptions of the three preparations (A–C) are given in the Experimental Section. Their respective pH-Stat recordings (for the digestions) are given to the right (together with pertinent details for dry weight of protein, amount of trypsin, pH of titration, and reaction mixture components). To the left are the corresponding Dowex 50-X2 column chromatograms (as recorded with a Spinco 120 or 120B instrument, 2.2- or 6.6-mm cuvet, with or without stream splitter). Details of the equivalent weight of preparation chromatographed, the developing systems, and the gradients used are presented in the figures (see also Experimental Section).

and 42,700 lacked significance.[7] In addition, the inherent errors in these determinations at relatively high densities and viscosities, as well as some degree of uncertainty in the extrapolation, provide an over-all uncertainty of probably no better than ±3000 on an absolute basis. Therefore, it may be concluded that within the experimental error, the completely dissociated polypeptide chains have one-half the molecular weight of the native enzyme. Finally, and in support of this conclusion that the native enzyme consists of two polypeptide chains of essentially identical molecular weights, may be cited the results of the high-speed equilibrium runs (according to Yphantis, 1964) where c_m (the concentration at the meniscus) had been reduced to almost zero and which permit, at least approximately and also within the large error of the procedure, an estimate of $\bar{M}_n$ (as well as of $\bar{M}_w$ and $\bar{M}_z$). In Figure 10, these runs in 0.1 M mercaptoethanol are presented for the native enzyme (at *ca.* 0.2 mg/ml), the enzyme in 8 M urea (at *ca.* 0.1 mg/ml), and the enzyme in 4 M guanidinium chloride (at *ca.* 0.6 mg/ml), from which the weight-average molecular weights ($\bar{M}_w$) and their estimated uncertainties could be calculated to be 85,000 ± 3000, 42,700 ± 1500, and 42,000 ± 1500, respectively. Also, for each run, the values for $\bar{M}_w$, $\bar{M}_z$, and $\bar{M}_n$, were essentially the same within the relatively large uncertainties of their estimations.

Chemical Studies on the Protein and Its Polypeptide Chains

Tryptic Digestions. For the data which are pertinent to this report, three types of preparations or derivatives are presented: (a) the performic acid oxidized protein; (b) the *S*-carboxymethylated protein, prepared by treating it with sodium borohydride in 0.7% lauryl sulfate, followed by *S*-carboxymethylation with iodoacetic acid (*cf.* Noltmann *et al.*, 1962); and (c) the *S*-carboxymethylated protein, prepared by treating it with mercaptoethanol in 8 M urea, followed by alkylation with iodoacetic acid (*cf.* Crestfield *et al.*, 1963). The results of comparatively small-scale tryptic digestions of these three preparations are given in Figure 11, in terms of their pH-Stat titrimetric recordings and the chromatographic separations of the tryptic peptides on Dowex 50-X2 (employing developing systems similar to those of Kimmel *et al.*, 1962). Although conversion of the 8 half-cystine residues/mole (*i.e.*, per 82,600 g) of protein (Noltmann *et al.*, 1962) was nearly complete in all three preparations (*viz.*, *ca.* 7.7 cysteic acid residues/mole for a, *ca.* 6.9 *S*-carboxymethylcysteine residues/mole for b, and *ca.* 7.7 *S*-carboxymethylcysteine residues/mole for c) nevertheless, the extents of digestion by trypsin, under these conditions, varied considerably. Thus, as predicted from the 101 lysine plus arginine residues estimated per 82,600 g (see below), the pH-Stat end points revealed that only in the case of preparation c were quantitative yields[8] of digested material approached. These yields were reflected in their chromatograms (note that the gradients for elution were slightly different for a than for b or c. Rechromatography of almost all of the fractions of b has been done on Dowex 1-X2 with developing systems similar to those of Schroeder *et al.* (1962), and many of the peaks have been found to be heterogeneous and to contain small yields of larger undigested peptides. It is apparently interesting that the treatment of the protein with lauryl sulfate (where the sample had been dialyzed prior to the digestion, but dialysis may be inefficient in the removal of lauryl sulfate) inhibits the attack by trypsin, possibly through binding of the lauryl sulfate (see above, under sedimentation velocity), or through retention of some ordered structure in the molecule. Only those results of the analyses of the tryptic peptides pertinent to this report will be described further and the results of digestion c will be emphasized, since, clearly, from the point of establishing identity or nonidentity of the polypeptide chains, the extent of digestion is of prime importance. Heterogeneous mixtures of carboxyl-terminal lysine or arginine tryptic peptides plus an undetermined number of larger and undigested fragments would make interpretations difficult. (However, the results of digestion b may prove of use later in the studies on the amino acid sequence since it contains a number of peptides, albeit in low yields, which can provide overlapping sequences.)

Digestion c contains a minimum number of undigested peptides, but the chromatogram reveals far less than the theoretical number of 101 tryptic peptide fractions expected for a single chain of 82,600 g. The results of a number of peptide maps (on digestion c) are given in Figure 12, where several reagents (in addition to ninhydrin; see Experimental Section) were employed to qualitatively identify a few different residues in the separated tryptic peptides. These data are summarized in Table II.

Column chromatography in all three cases had revealed the presence of relatively large amounts of free lysine and free arginine (identified after rechromatography and amino acid analysis) in the tryptic digestions, which amounted to (in case c, Figure 11) *ca.* 3 lysines and 2 arginines per 41,300 g of protein (estimated by comparison with standards of lysine and arginine, chromatographed under identical conditions); thus, there would appear to be lysyllysine or arginyllysine, and arginylarginine or lysylarginine sequences within the polypeptide chains. Only 45–50 lysine- plus arginine-containing peptides and free arginine and lysine could be detected in all the peptide maps. A total of 48 peptides would appear to be present in reasonable yields, with two present in such trace yields that they possibly represent larger and undigested fragments. Thus, only approximately half of the theoretical number of tryptic peptides are obtained in

[7] A decrease of 0.01 cc/g in V would amount to a decrease in $\bar{M}_w^0$ of 2300 in the extrapolated value.

[8] A quantitative estimation of percentage hydrolysis is permitted only with the assumption of a mean pK' for the liberated α-amino groups; *e.g.*, with a pK' of 7.4 (Richards, 1955), the percentage hydrolysis would be calculated as: 48, 96.5, and 101% for b, a, and c, respectively.

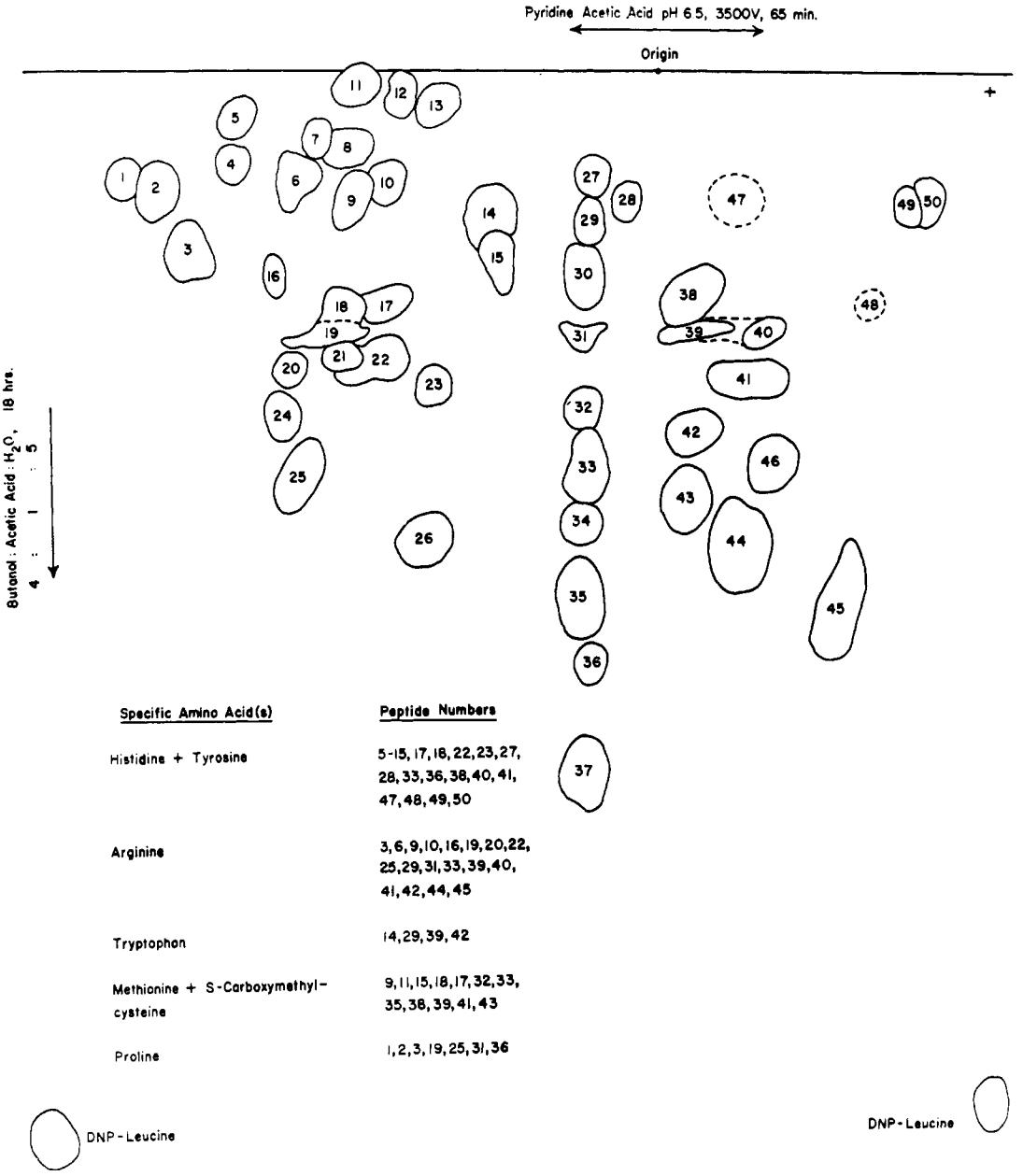


FIGURE 12: Tryptic peptide map of *S*-carboxymethylated ATP–creatine transphosphorylase (alkylated in 8 M urea). Vertical direction: paper chromatography with butanol–acetic acid–water (4:1:5) for 18 hr; horizontal direction: electrophoresis at 3500 v for 65 min, in pyridine–acetic acid (pH 6.4). The map represents a composite drawing of several runs, which were stained individually for amino acids listed in the figure, or with ninhydrin. The ninhydrin-positive peptide "areas" are arbitrarily numbered (those in dashed areas gave exceedingly faint ninhydrin stains); the respective amino acid containing peptides (other than lysine or arginine) which were detected in each of the areas are indicated by their respective number. The point of application (of *ca.* 1.5 mg of equivalent protein) for the electrophoresis was made slightly off center, and indicated by "origin," in order to resolve the cathodic components. DNP-leucine was added as a marker for the chromatography, on either side of the "map."

reasonable yield. In Table III, the amino acid composition of the protein is presented, taken from Noltmann *et al.* (1962) originally calculated in terms of residues/81,000 g. These data are now recalculated in terms of residues/82,600 g, residues/41,300 g, and nearest integral amino acid residues for hypothetical half-molecules of mol wt 41,300. The 51 lysines plus arginine residues per polypeptide chain of 41,300 g (and the fact that lysine is the carboxyl terminus; see below) would be expected to yield 51 tryptic peptides, if both polypeptide chains were extremely similar (or identical) in composition, or 48 peptide map

TABLE II: Summary of Tryptic Peptide Maps of *S*-Carboxymethylated ATP–Creatine Transphosphorylase.

Amino Acid Residues	Peptides Found No.	Range[a]	Reagent	No. of Peptides Calcd from Nearest Integral Residues/ 41,300 g
Lysine + arginine	48	45–50	Ninhydrin	51 (−3)[b]
Arginine	18	14–18	Sakaguchi	18 (−1)[b]
Histidine + tyrosine	26	26–30	Pauly	27
Methionine + *S*-carboxymethylcysteine	12	12–13	Platinic iodide	14
Tryptophan	4	3–5	Ehrlich	4

[a] The number of areas found on several separate peptide maps, run under the same conditions. [b] Free lysines (3) and free arginines (2) per 41,300 g are liberated in tryptic digest (indicative of lysyllysine or arginyllysine, and arginylarginine or lysylarginine sequences in the subunit polypeptide chains).

"areas" after correction for the liberated free lysine and arginine (which would thus merge as two single areas).[9]

Also, the 18 arginines/41,300 g (Table III) would be expected to yield 17 areas (Table II), and a range of 14–18 was found, with 18 being most likely. Similarly, 26–30 histidine- plus tyrosine-, 12–13 methionine- plus *S*-carboxymethylcysteine-, and 3–5 tryptophan-containing peptides may be compared with the theoretical values at 27, 14, and 4, respectively; traces of undigested material make it important to quote these as ranges; the most likely values in terms of yields are given in Table II. (See also the Experimental Section for qualifying remarks in regard to the use of some of these reagents for identification of the several amino acid residues.)

These analyses of the tryptic peptides would point heavily to an identity of the two polypeptide chains. However, only a complete determination of the amino acid sequences of all the isolated tryptic peptides will establish this point. There is, of course, the inherent danger in such preliminary and qualitative analyses that the two chains may be very similar, but differ only slightly in the sequence of a few amino acid residues, and only an evaluation of the total amino acid sequence of the protein (which is in progress) will ultimately establish the identity or nonidentity of these two polypeptide chains.

Carboxyl-Terminal Group Analysis. Olson and Kuby (1964) had found that, at alkaline pH, carboxypeptidase A could cleave carboxyl-terminal lysine from this protein and had established the carboxyl terminal sequence as glutaminyllysine. This reported specificity of carboxypeptidase A appeared to be unique at the time, but it has since been confirmed by Grosskopf *et al.* (1966) for the case of hemerythin and tryptic peptides derived from it. However, the rates of digestion of the native enzyme by carboxypeptidases were extremely slow, and a significant error could have been introduced in establishing the end point of the digestion, which appeared to average *ca.* 1.06 moles of lysine/81,000 g of protein (with a range of 1.00–1.13). Thus, it was important to confirm the stoichiometry by other techniques and certainly such a value would be difficult to correlate with the present results.

CARBOXYPEPTIDASE B digestions were selected because of the ease and rapidity with which it can catalyze the hydrolysis of carboxyl-terminal basic residues from peptides (Folk *et al.*, 1960). The reaction proved to be extremely rapid with DFP-treated carboxypeptidase B at mole ratios of *ca.* 1:40 (carboxypeptidase B to native protein) and was essentially complete after about 0.5 hr at 30° (Figure 13). Only lysine could be detected in the reaction mixtures, after subtraction of minute traces derived from carboxypeptidase B controls, and a stoichiometric yield of *ca.* 2.0 moles of lysine liberated/82,600 g of protein was obtained. Even carrying the reaction up to 300 min did not change the yield, nor liberate any other amino acids in significant yields. As shown in the upper graph of Figure 13, the carboxypeptidase B retained its activity under these conditions for the time duration of these experiments. It is interesting that glutamine (the penultimate amino acid residue (Olson and Kuby, 1964)) was not released, indicative of a resistant site to carboxypeptidase B attack, immediately preceding glutamine.

HYDRAZINOLYSIS based on procedures of Akabori *et al.* (1956) and Fraenkel-Conrat (Funatsu *et al.*, 1964; Niu and Fraenkel-Conrat, 1955) was utilized to confirm these observations with carboxypeptidase B. Although the yields of carboxyl-terminal lysine by this technique have usually been low (*cf.* Niu and Fraenkel-Conrat, 1955) and rarely exceeded 30–40%, nevertheless, it appeared likely that these yields could be increased by employing a lower temperature for the hydrazinolysis than ordinarily used and thereby reducing the rate of degradation of the liberated lysine in hydrazine. In agreement with the results of Korenman *et al.* (1966) (who used an unpublished procedure of Bennett and W. J. Dreyer) on a β-galactosidase, at lower temperatures the half-life of free lysine in hydrazine is considerably increased and at 85° in hydrazine, its degradation appeared to follow pseudo-first-order kinetics with a $t_{1/2}$ calculated to be about 93 hr. Preliminary tests (see Experimental

[9] For the area on the peptide map represented by lysine, three residues would have merged as one, and for the arginine "area," two residues would have merged as one; therefore, the total number of expected areas would be 51 − (2 + 1) = 48.

TABLE III: Amino Acid Composition of ATP–Creatine Transphosphorylase.

Amino Acid Residue	Amino Acid Residues/81,000 g of Protein[a]	Amino Acid Residues/82,600 g of Protein	Amino Acid Residues/41,300 g of Protein	Nearest Integral No. of Amino Acid Residues/ 41,300 g of Protein
Aspartic acid	83.06	84.70	42.35	42
Threonine	33.92	34.59	17.30	17
Serine	41.24	42.05	21.03	21
Glutamic acid	74.48	75.95	37.98	38
Proline	36.55	37.27	18.64	19
Glycine	62.35	63.58	31.79	32
Alanine	25.34	25.84	12.92	13
Valine	53.03	54.08	27.04	27
Methionine	18.99	19.37	9.69	10
Isoleucine	25.34	25.84	12.92	13
Leucine	69.90	71.28	35.64	36
Tyrosine	18.82	19.19	9.60	10
Phenylalanine	29.92	30.51	15.26	15
Lysine	64.98	66.26	33.13	33
Histidine	33.35	34.01	17.05	17
Arginine	34.38	35.06	17.53	18
Tryptophan	7.29	7.43	3.72	4
Total cystine (half)[b]	7.66	7.81	3.91	4
Total				369

[a] Taken from Noltmann *et al.* (1962). [b] Taken from this report; represents the over-all mean of six determinations of *S*-carboxymethylcysteine (after 20-hr acid hydrolysis and corrected for 4% hydrolytic loss) on two samples of *S*-carboxymethylated protein with IAA (see Experimental Section).

Section) on a lysine-containing pentapeptide of known sequence made it seem very likely that the method could be successfully applied to ATP–creatine transphosphorylase, and that suitable corrections could be made for degraded lysine (see Experimental Section). Thus, in Figure 14 (lower plot) the results are presented in terms of a plot of moles of lysine recovered/82,000 g of protein, as a function of time of hydrazinolysis. The upper plot of Figure 14 depicts the first-order plot of free lysine under these conditions and provides the corrections for the measured values. From the corrected curve, one may estimate that after 70 hr, 1.9 moles (±0.2) of lysine had been liberated/82,600 g of protein, in good agreement with the carboxypeptidase B digestions. Small amounts of what appeared to be glycine, threonine, serine, leucine, and isoleucine were found in the reaction mixtures, but it is a common finding that breakdown of their respective hydrazides yields trace amounts of these amino acids (see Experimental Section). From the uncorrected curve, at its maximum, one may estimate that a yield of *ca.* 60–65% of carboxyl-terminal lysine could be obtained by this procedure, and apparently this yield exceeds that of the older procedures (Niu and Fraenkel-Conrat (1955); but see Braun and Schroeder (1967) for a reexploration of this technique, and compare Locker (1954)), and is in accord with the yields of Korenman *et al.* (1966) on β-galactosidase. Agreement with values derived by insertion of an internal standard also provided some support for the reliability of procedure. Finally, one may calculate from the corrected curve a $t_{1/2}$ of about 23 hr for formation of free lysine from the protein. The kinetics of hydrazinolysis appear to roughly approximate that of a series of two pseudo-first-order reactions, as described very approximately by

$$\text{protein} \xrightarrow[\text{(hydrazine)}]{k = 3\times10^{-2}\ \text{hr}\ (\text{formation})} \text{lysine} \xrightarrow[\text{(hydrazine)}]{k = 0.75\times10^{-2}\ \text{hr}\ (\text{degradation})} \text{degradation products}$$

(*cf.* also Akabori *et al.*, 1956). The fourfold ratio in the rate constants ($k_{\text{formation}}/k_{\text{degradation}}$) makes the procedure practicable. Doubling this ratio would have considerably increased the experimental yield, but although a further decrease in temperature might permit this, it might also make the rate of formation of free lysine so extraordinarily slow as to render the method unfeasible.

In conclusion, both procedures now yield 2 carboxyl-terminal lysines/82,600 g of protein. This fact, coupled

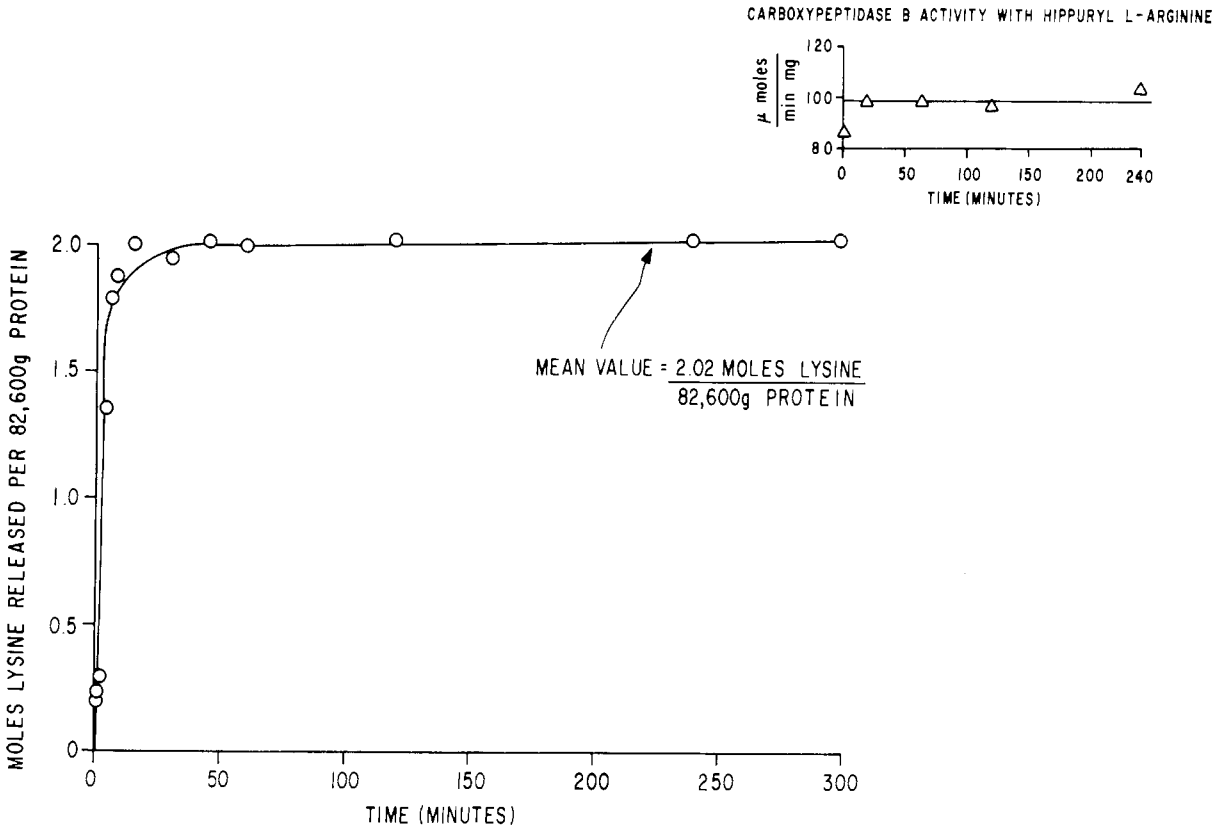


FIGURE 13: Kinetics of carboxypeptidase B catalyzed hydrolytic reaction with ATP–creatine transphosphorylase in 0.025 M borate (Na^+) (pH 9.02) at 30°. The upper right-hand insert shows the activity of carboxypeptidase B under these conditions. The lower, left-hand plot provides all the data measured from 0 to 300 min (21.33 mg of protein (or 0.258 μmole; $\overline{M}_w$ = 82,600) in 1.00-ml reaction volumes containing 0.0064 μmole of carboxypeptidase B, or a mole ratio of *ca.* 40:1, substrate protein to carboxypeptidase B), and are recalculated in terms of moles of lysine released/82,600 g of protein (see Experimental Section for details).

with the analyses of the tryptic peptides and the demonstration that the protein may be dissociated into two polypeptide moieties of essentially the same molecular weight, would make it appear highly unlikely that the two component polypeptide chains of the protein are very much dissimilar in composition.

Over-All Discussion and Summary

The physical and chemical evidence presented here (and summarized in Table IV for those data which may be conveniently tabulated) demonstrates that the native, compact, and ordered enzyme molecule contains two polypeptide moieties, each of which has the same molecular weight within experimental error and each of which appears to have carboxyl-terminal lysine. Moreover, these two polypeptide components do not appear to be held together, within the ordered structure of the native molecule, by any covalent linkages; interpolypeptide disulfide linkages have been excluded.

The nature of the binding sites between the polypeptide component parts of the molecule, however, are such that they must involve a summation of a large number of forces so sufficiently strong as to make detection of any degree of dissociation in the native structure well beyond the sensitivity of the techniques employed here. Thus, if one were to calculate the degree of dissociation, from the guanidine solutions extrapolated to zero guanidine, in a manner similar to that of Tanford and his associates (Kawahara *et al.*, 1965) for the hemoglobin system, then at an ionic strength of 0.16, it would correspond to less than 0.009; admittedly, however, unfolding (see above) in guanidine solutions makes such calculations uncertain. Also, qualitatively, one might suppose that since high salt (1.4 M NaCl–0.1 M mercaptoethanol) does not appear to be effective in disrupting the enzyme (where the $s_{20,w}$ had decreased from 5.2 to only *ca.* 4.7 S at *ca.* 8-mg/ml total protein concentration; see footnote 4), ionic forces between subunits would be deemphasized, and possibly hydrophobic forces together with superimposed interchain (and possibly intrachain) hydrogen bonds are the likely contributors to the over-all and relatively enormous summation of forces involved in maintaining the

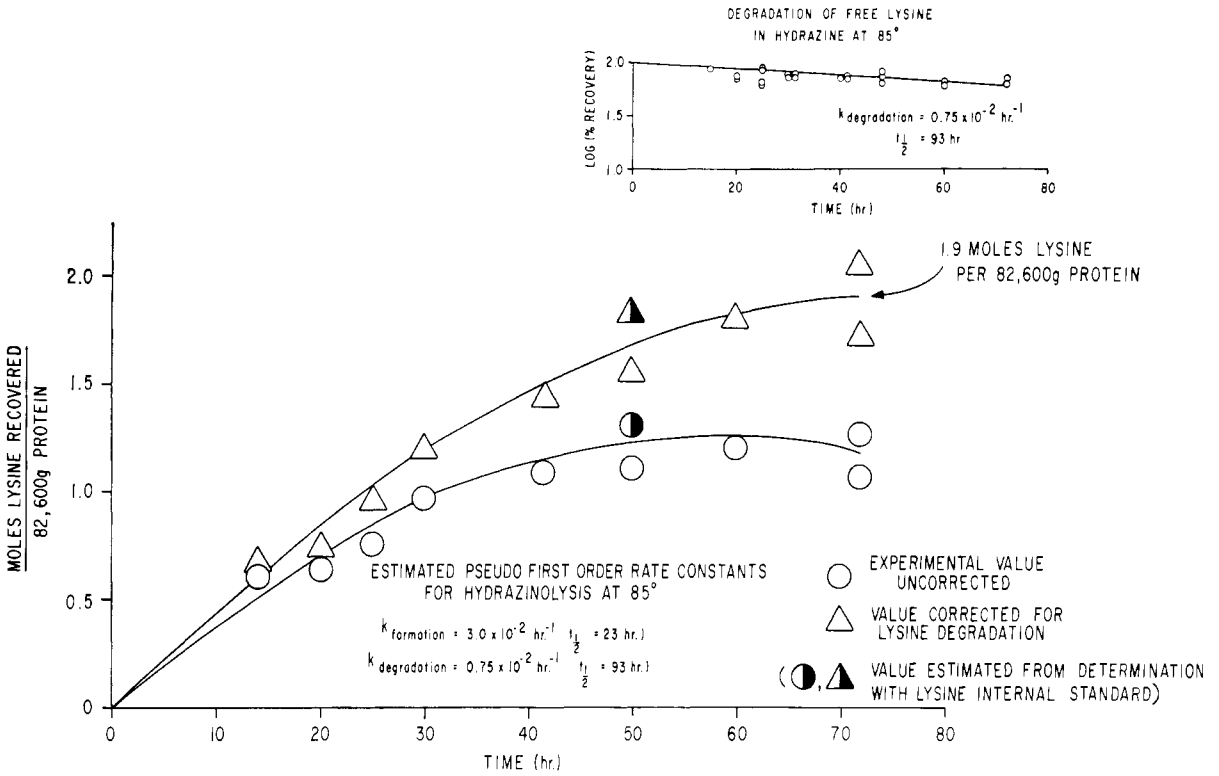


FIGURE 14: Kinetics of hydrazinolysis of ATP–creatine transphosphorylase at 85°. The upper right-hand insert shows the kinetics of degradation, in hydrazine, of free lysine (0.155 μmole in 0.5 ml of hydrazine), at 85°, in terms of a first-order plot ($k_{degradation} \cong 0.75 \times 10^{-2}$ hr^{-1}; $t_{1/2} \cong 92$ hr). The lower plot provides the experimentally measured values of moles of lysine recovered/82,600 g of protein (○——○, values obtained from 11.32 mg of protein in 0.5 ml of hydrazine and includes a value (◑) estimated after insertion of an internal standard of lysine). The upper curve, indicated by Δ——Δ, represents values corrected for degradation of free lysine at each time value (and estimated from the first-order degradation kinetics, upper right-hand insert). From the corrected plot (Δ——Δ), which includes one point ◭, estimated from the value which had an internal standard, a $k_{formation} \cong 3.0 \times 10^{-2}$ hr^{-1}, or $t_{1/2} \cong 23$ hr, may be approximated for the liberation of C-terminal lysine at 85°.

tightly knit structure of the native enzyme molecule ($f/f_0 = 1.21$). It is of course an attractive hypothesis to presume that at low concentrations of urea, guanidine, or lauryl sulfate, a process of dissociation is initiated into the ordered polypeptide moieties which have approximately the same frictional ratio as the native molecule (perhaps as a result of imbibition of solvent components; see discussion under lauryl sulfate) and then at higher concentrations of denaturants, especially guanidine hydrochloride, there is a transition to an uncoiled disorganized state (see discussion following Figures 4 and 5), and the equilibria are eventually shifted quantitatively toward this state of maximum entropy. However, this hypothesis cannot be established from these data alone and a more cautious postulate would suppose that an intermediate state of aggregation is involved in the process of denaturation (see Figures 4 and 6, and *cf.* Westhead, 1964) and that dissociation and denaturation in guanidine or urea proceed fairly closely together or coincidently. A very early and isolated observation on the remarkable enzyme stability in aqueous ethanol (at dilute ionic strength) (Kuby *et al.*, 1954) must also be allowed for in considerations of the forces involved in the intact molecule.

Finally, although a systematic study on reversibility of the denaturation process has not been undertaken, a single observation on this point would be pertinent to this discussion. A solution of protein of 2.8 mg/ml in 4 M guanidine hydrochloride (0.1 M mercaptoethanol, pH 6.8) was kept at 20° for 8 hr and then dialyzed at 3° for 95 hr against repeated charges of 0.15 M KCl–0.01 M Tris (pH 7.8). Approximately 57% of the total protein was recovered in soluble form, with a specific enzyme activity which had decreased also to *ca.* 55% of its original value. Thus, even under conditions where the enzyme had been subjected to such severe denaturing conditions, which were sufficient to cause complete dissociation and unfolding of the polypeptide chains, some sort of annealing and re-formation (perhaps dictated by the amino acid sequence; see Schachman, 1963) to a structure approaching the original enzyme would appear to have taken place (see also Dawson *et al.*, 1965, 1967, for a study of hybridization

TABLE IV: Physical and Chemical Properties of ATP–Creatine Transphosphorylase and Its Component Polypeptide Chains.

A. Kinetic Unit (Native Enzyme)	
$s^0_{3°,b}$, sedimentation coefficient ($\bar{c} \rightarrow 0$, pH 7.8), sec	3.30×10^{-13}
$s^0_{20,w}$, sedimentation coefficient ($\bar{c} \rightarrow 0$, pH 7.8), sec	5.31×10^{-13}
$s_{20,w}$, sedimentation coefficient (8.2 mg/ml, pH 7.8, with RSH[a]), sec	5.11×10^{-13}
$s_{20,w}$, sedimentation coefficient (7.6 mg/ml, 1.4 M NaCl, pH 6.3 with RSH), sec	4.72×10^{-13}
$D^0_{3°,b}$, diffusion coefficient ($\bar{c} \rightarrow 0$, pH 7.8), cm²/sec	3.58×10^{-13}
$D^0_{20,w}$, diffusion coefficient ($\bar{c} \rightarrow 0$, pH 7.8), cm²/sec	6.10×10^{-7}
$M^0_{s,D}$, molecular weight by sedimentation and diffusion ($c \rightarrow 0$)	82,700 ± 1600
M_{eq}, molecular weight by sedimentation equilibrium, g/mole	82,600 ± 2300
$\bar{M}_z/\bar{M}_w$	1.005
$\bar{V}$, partial specific volume from amino acid composition,[b] cm³/g	0.735
$\bar{V}^0_{3°}$, partial specific volume at 3° ($c \rightarrow 0$)[c]	0.736
$\bar{V}^0_{20°}$, partial specific volume at 20° ($c \rightarrow 0$)[c]	0.744
f/f_0, frictional ratio	1.21 (a/b = 4.4)
$(\Delta n/\Delta c)_{3°}$, refractive index increment (λ 546 mμ) (g/100 ml)$^{-1}$	1.84×10^{-3}
C-terminal groups, by hydrazinolysis, Lys/mole	1.9 ± 0.2
C-terminal groups, by carboxypeptidase B, Lys/mole	2.0
B. Polypeptide Chains (Noncovalently Linked)	
$\bar{M}^0_w$, weight-average molecular weight ($c \rightarrow 0$, 4 M guanidine hydrochloride–0.1 M RSH, pH 6.3)	42,700 ± 1300
$\bar{M}_w$, weight-average molecular weight (2.5 mg/ml, 0.001 M EDTA-4 M guanidine hydrochloride, *without* RSH, pH 6.3)	40,200
$\bar{M}_w$, weight-average molecular weight (0.1 mg/ml, 8 M urea–KCl–phosphate–EDTA–RSH, pH 6.8)	42,700
$s_{20,w}$, sedimentation coefficient (8 mg/ml) (sec)	
(1) In 4 M guanidine hydrochloride–EDTA–RSH, pH 6.3	1.44×10^{-13}
(2) In 8 M urea–KCl–phosphate–EDTA–RSH, pH 6.8	1.79×10^{-13}
(3) In 0.035 M lauryl sulfate–NaCl–EDTA–RSH, pH 6.3	2.48×10^{-13}
Tryptic peptides found	Approximates theoretical no./41,300 g

[a] RSH, 2-mercaptoethanol. [b] Taken from Noltmann *et al.* (1962). [c] Extrapolated from the data of Noda *et al.* (1954).

experiments conducted in denaturants). Further, the native enzyme is resistant to some degree to the disruptive influence of 4 M urea, as the results at 3° imply (in 0.01 M Tris–0.15 M KCl (pH 7.8)–0.1 M mercaptoethanol; see Figure 6), and only in 8 M urea after periods of time at 20° did complete disruption take place. The resistance to tryptic digestions of the native enzyme (unpublished observations, S. A. Kuby) and of the lauryl sulfate treated *S*-carboxymethylated protein (see Figure 11), and the times for digestion required for the 8 M urea-treated *S*-carboxymethylated protein, also point to a molecular structure held together with unusual strength and with a high degree of order.

The analyses for total half-cystine residues (see Noltmann *et al.*, 1962, and *cf.* the present results in Table III) have clearly pointed to 8 half-cystine residues/82,600 g of protein or 4/subunit. However, since only 6.3–6.5 SH groups/81,000 g were amperometrically titratable with silver in 8 M urea (Benesch *et al.*, 1955; Noltmann *et al.*, 1962) it had been postulated (Noltmann *et al.*, 1962) that there might be a disulfide bridge within the molecule. This postulate, however, would be incompatible with the idea that there are four half-cystines per polypeptide chain and no disulfide bridges between chains. Thus, one is forced to conclude that only 3.15–3.25 cysteinyl residues/81,000 g (or 3.21–3.32/82,600 g) were titrated with silver in 8 M urea, out of a total of four SH groups per chain, and that either quantitative titration in 8 M urea was precluded by some residual interactions still remaining between chains, or that a statistical portion (of *ca.* 0.7–0.8 SH group/chain) was oxidized during or just prior to the titration in 8 M urea; this problem would bear reinvestigation.

All the information presented here will lay a good foundation for studies on the primary sequence of this protein, a problem which is now greatly simplified by the fact that it consists of two noncovalently linked polypeptide chains of similar molecular weight. The identity or nonidentity of these subunits will be ultimately resolved by such studies, but certainly there

is now no evidence which would run in contradistinction to the proposed dimeric structure for this protein (Dawson *et al.*, 1965, 1967), and all the information presented here would tend to support this conclusion which had been reached independently by Kaplan and associates (Dawson *et al.*, 1965, 1967), primarily from hybridization experiments.

Further studies on the mechanisms of denaturation are to be expected to yield information on the three-dimensional structure of the intact two-chain molecule in solution, and since the over-all goal is the elucidation of its ordered geometrical structure, probings into any area designed to shed light on the structure of the chains themselves would be of aid. Only a final consideration of the over-all molecule will permit a correlation of the structure of this enzyme with its catalytic properties. Kuby *et al.* (1962) had assigned two binding sites for the nucleotide substrates to this enzyme, and both binding sites appeared to be equivalent and with little interaction between them, and it now seems reasonable to presume that there is one binding site per polypeptide chain. (Compare, also, the studies on the two reactive SH groups per mole in this protein (Mahowald *et al.*, 1962a; Watts *et al.*, 1961; Thomson *et al.*, 1964; Mahowald, 1965)). Whether there are any interactions between catalytic sites, however, is an interesting area for future kinetic studies.

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