Studies on Adenosine Triphosphate Transphosphorylases. VIII. Homogeneity and Physicochemical Properties of the Crystalline Adenosine Triphosphate–Creatine Transphosphorylase from Calf Brain*

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ABSTRACT: This crystalline protein has withstood several criteria of purity, *e. g.*, sedimentation velocity, sedimentation equilibrium, liquid-boundary electrophoresis, electrophoresis on cellulose acetate, and polyacrylamide disc electrophoresis, provided certain "instability" problems are recognized. The molecular weight by sedimentation equilibrium and the value for $s^0_{20,w}$ of the calf brain have been assigned; and apparently these values are only slightly higher than those reported for the rabbit muscle adenosine triphosphate–creatine transphosphorylase (Yue, R. H., Palmieri, R. H., Olson, O. E., and Kuby, S. A. (1967b), *Biochemistry 6, 3204*). Similar to the rabbit muscle enzyme, the calf brain enzyme appears to consist of two noncovalently linked polypeptide chains; a conclusion deduced by sedimentation equilibrium studies conducted in 4 M guanidinum chloride, in the presence and absence of 2-mercaptoethanol. Interestingly, the calculated frictional ratios appear to be identical for both calf brain and rabbit muscle enzymes, which might imply a similar over-all gross molecular shape, in solution. However, the electrophoretic mobilities (liquid boundary and on supporting media) have revealed large differences in the isoelectric points for the calf brain *vs.* calf muscle isoenzyme (pI_0 = 5.6 *vs.* 7.3, at zero ionic strength), indicative of significant differences in their charge distributions. Coincident with the electrophoretic studies on the calf isoenzymes, the contrast between the more stable molecular unit to be found in the muscle enzyme *vs.* the brain enzyme has become evident.

In the previous paper of this series (Keutel *et al.*, 1968), the isolation of the crystalline ATP–creatine transphosphorylase from calf brain was described. This report will deal in particular with some studies which bear on the homogeneity of this protein preparation, with an estimation of its molecular weight, and with an evaluation of its subunit structure as revealed from physical measurements. Coincident with this work, several physicochemical properties of the isolated enzyme have been determined or assigned. Within this report, a quantitative comparison of only a single physical property of the calf brain enzyme with its crystalline calf muscle isoenzyme (Jacobs *et al.*, 1968) will be given, and which is pertinent to this report, *viz.*, that of their electrophoretic behavior. A comparison with the rabbit muscle enzyme's subunit structure (Yue *et al.*, 1967b) is also drawn. Further physical comparisons and contrasts with the crystalline ATP–creatine transphosphorylases which have been isolated from the brain and muscle of man, calf, and rabbit will be postponed to future communications, wherein it is hoped a final analysis may be presented which incidentally may be of intrinsic interest also from a comparative biochemical standpoint. A preliminary report of some of this work has been given elsewhere (Jacobs *et al.*, 1968).

Experimental Procedure

Materials. Crystalline ATP–creatine transphosphorylase from calf brain was isolated by the procedure described by Keutel *et al.* (1968). All reagents used for buffers were the best available analytical grade commerical products. 2-Mercaptoethanol was redistilled *in vacuo*; guanidine hydrochloride (Eastman) was purified by the procedure of Nozaki and Tanford (1967). Twice-distilled, deionized water was used in the preparation of all solutions for the physical measurements (when necessary, any dissolved traces of CO_2 were removed by boiling). The reagents used for disc electrophoresis were obtained from Canalco or Eastman; and the cellulose acetate strips (Sepraphore III) and Ponceau S dye, from Gelman. The enzymes (hexokinase and glucose 6-phosphate dehydrogenase), substrates (ADP, creatine phosphate, glucose, and TPN^+), and dyes (phenazine methosulfate and nitrotetrazolium blue) used for the coupled enzyme-catalyzed stains for ATP–creatine transphosphorylase on cellulose acetate or polyacrylamide gels were products of Boehringer or Sigma.

Methods

Sedimentation Velocity and Sedimentation Equilibria

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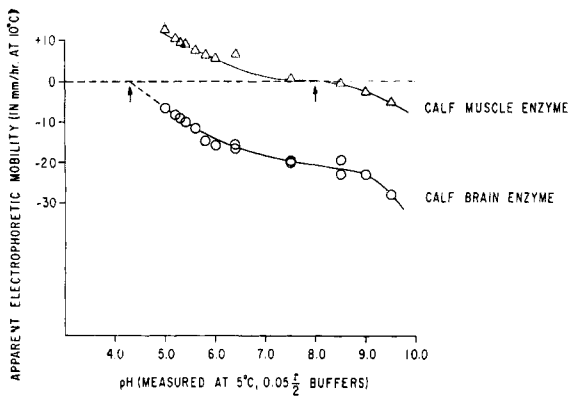

FIGURE 1: Electrophoresis on cellulose acetate (Sepraphore III), at an average temperature of 10°, of crystalline ATP-creatine transphosphorylase from calf brain and from calf muscle. The following buffers employed (containing 0.001 M β-mercaptoethanol) were all 0.05 M in total buffer species adjusted to a total ionic strength of 0.05 with KCl: for pH values 9.5–8.5, Tris (HCl); pH 7.5–6.4, imidazole (HCl); and pH 6–5.0 sodium acetate (acetic acid). Runs were performed at an applied volume of 150 V, 10–7 mA, for 30–60 min. Apparent mobilities were measured from densitometric tracings and expressed in arbitrary units of mm/hr. (△——△) Calf muscle enzyme; (○——○) calf brain enzyme. Arrows denote estimated pH values of zero mobility: approximately 7.8 and 4.5 for calf muscle and calf brain enzyme, respectively.

Measurements. Samples of the enzyme were dialyzed (with repeated changes) against freshly prepared solutions containing 0.15 M KCl, 0.01 M Tris, 0.001 M EDTA, and 0.1 M β-mercaptoethanol (pH 7.8). Sedimentation velocities were measured, with Epon-filled double-sector 12-mm cells, at 3° in a Spinco Model E ultracentrifuge equipped with an RTIC temperature-control system and phase plate as schlieren diaphragm (Yue *et al.*, 1967c). Sedimentation coefficients at 3° and buffer ($s'_{3°,b}$) were calculated in the usual fashion from log x *vs.* t plots (Schachman, 1957) and converted into $s^0_{20,w}$ values (Svedberg and Pedersen, 1940) with the use of measured values for the density and viscosity of the buffer employed. The partial specific volumes extrapolated from the data of Noda *et al.* (1954) for the rabbit skeletal muscle enzyme to zero concentrations at 3 or 20° were assumed to apply for the native calf brain enzyme.

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). A 12-mm double-sector synthetic boundary cell of the capillary type with sapphire windows was employed and Kodak IIG spectroscopic plates were used to record the interferograms. The experimental procedure followed that described previously[1] with slight modifications (Yue *et al.*, 1967a). In the experiments with protein solutions containing 4 M guanidine hydrochloride at 20°, the preparation of the protein solutions and manipulative procedures were similar to that employed (Yue *et al.*, 1967b) for the rabbit muscle enzyme, with each sector filled with the same volume (137 μl) to match the menisci, as discussed by Schachman and Edelstein (1966). 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 (Svedberg and Pedersen, 1940). The legends to the figures provide 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 $M_{w,x} = [2RT/\{(1 - \bar{V}\rho)\omega^2\}]\mathrm{d}(\ln J_x)/\mathrm{d}(x^2))$, and the mean z-average molecular weight for the entire cell contents calculated from the limiting slopes of the ln J_x *vs.* X^2 plots at the cell bottom and 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 have been described (Yue *et al.*, 1967a). The checks employed to establish the reliability of the interferometric procedure for sedimentation equilibrium analyses at high guanidine hydrochloride–β-mercaptoethanol concentrations have been delineated for several protein systems and applied successfully to the rabbit muscle enzyme (Yue *et al.*, 1967b). For the protein solutions in 4 M guanidine hydrochloride a $\bar{V}$ = 0.735 cm³/g was employed (the same value as utilized for the rabbit muscle enzyme (Yue *et al.*, 1967b) and which had been calculated from its amino acid composition (Noltmann *et al.*, 1962)).

The interferometric technique of sedimentation equilibrium in highly dilute protein solutions at relatively high speeds according to Yphantis (1964) (*i.e.*, the meniscus depletion technique) was also utilized in this study; and the precautions and experimental technique followed that of Yphantis (1964) as described (Yue *et al.*, 1967b). Evaluation of molecular weight averages ($\bar{M}_n$, $\bar{M}_w$, and $\bar{M}_z$) was also made according to the procedures of Yphantis (1964). The larger uncertainties in this technique compared with the conventional low-speed equilibrium technique have been noted (Yue *et al.*, 1967b) and will be further indicated below by the statistical plots.

Liquid-Boundary Electrophoresis. Moving-boundary electrophoretic measurements were conducted in the Spinco Model H instrument at 1.0° with the 2-ml micro-Tiselius cell and schlieren (as well as Rayleigh) optics. Solutions of protein (in this case either of the crystalline calf brain or of the crystalline calf muscle enzyme) were exhaustively equilibrated by dialysis against freshly prepared buffers at various pH values and ionic strengths. In the case of the calf brain enzyme, 2-mercaptoethanol was included in the buffer to stabilize the enzyme during dialysis and electrophoresis, but was omitted for studies on the calf muscle enzyme. Conductivity measurements at 0.0° were made on the equilibrated buffer, with the use of a Radiometer conductivity meter DCM 2d and a refrigerated bath (Precision Scientific Instruments, Temptrol bath). The calculated electrophoretic mobilities are consequently those for 0° (Tiselius, 1937). Migration of the boundaries was measured from the center line of the boundary with a micro-

[1] Spinco Division Manual, technical manual no. E-IM-3, 1964.

comparator (Gaertner) and calculation of mobilities was made in the conventional manner (Abramson *et al.*, 1942; Longsworth, 1942) on the descending boundary. To minimize deviations from ideality and convective disturbances in the cell, relatively dilute protein concentrations (1–5 mg/ml) and comparatively low electric field strengths (which, except in two runs, did not exceed *ca.* 4.5 V/cm) were employed for the 0.10 ionic strength buffers and the 2-ml cell. For most cases, the patterns for both calf brain and calf muscle enzyme appeared enantiographic and symmetrical, except in the region of instability (at pH value less than 6) of the calf brain enzyme. The pH values of the buffer were measured at 3° with a Beckman research meter sensitive to 0.001 pH unit.

Electrophoresis on Supporting Media. Electrophoresis on cellulose acetate was conducted (average temperature 10°) on Sepraphore III strips with the use of a Gelman chamber 51101 (refrigerated) and a Spinco Duostat power supply. Strips were stained for protein with ponceau S (in 0.5% trichloroacetic acid), washed, cleared and densitometrically analyzed with the use of a Scan-A-Tron attachment for the Spinco Analytrol densitometer.[2] Effects due to electroendosmosis seemed to be absent as checked by the use of the uncharged Blue Dextran macromolecule (Pharmacia). Reproducibility of the apparent electrophoretic mobility values was good provided electrophoretic runs were performed on strips from an identical lot; but small differences could be observed under otherwise identical conditions, between different lots (*e.g.*, no. 1486 and 1518) which seemingly were due to a varying, but slight degree of adsorption or retention of the protein. Detection of the enzyme activity on the strips after the runs were made by application (*via* another overlying strip) of a coupled enzyme–dye reaction mixture,[3] developed at 37° and fixed in 7% acetic acid. Coincidental with these measurements, adenylate kinase activity was shown to be absent in both crystalline calf brain and calf muscle preparations, by staining the strips with a suitable reaction mixture.

The Canalco Model 6 apparatus (refrigerated) and system were employed for polyacrylamide gel disc electrophoresis (Davis, 1964; Ornstein, 1964) with the slight modifications described[4] for "standard" runs at a discontinuous alkaline pH gradient. However, use of the "standard" 7% polyacrylamide gel led to serious artifacts, to multibanded patterns, and to a relatively large percentage of denatured and aggregated protein, when the gel had been polymerized with persulfate, and which apparently was due to persulfate oxidation. Therefore, photopolymerization with riboflavin (Davis, 1964) was utilized for preparation of the 7% gels.

Gels were stained overnight for protein in 0.5% Naphthol Blue Black in 0.7% acetic acid and destained electrophoretically.[4] Enzymatic activity was detected by transferring the gels to reaction mixtures similar to those used for cellulose acetate.[3]

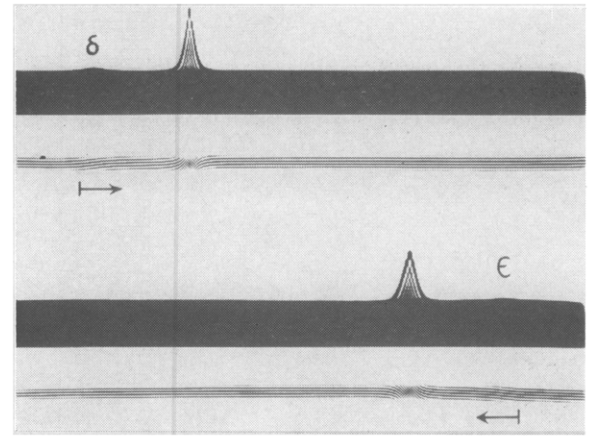

FIGURE 2: Liquid-boundary electrophoresis patterns at 1.00° of crystalline ATP–creatine transphosphorylase from calf brain as measured in 0.066 M imidazole (HCl)–0.010 M β-mercaptoethanol, Γ/2 = 0.050, pH 7.02 (measured at 3°). The initial protein concentration was 3.5 mg/ml, and the potential gradient 4.1 V/cm. The bases of the arrows indicate the approximate starting position of the boundaries, and the false boundaries, ϵ and δ, are indicated for the descending (lower pattern) and ascending (upper pattern) limbs, respectively. Beneath the schlieren pattern of each limb, the respective Rayleigh interferometric patterns are also presented. Upper patterns: ascending limb, photograph taken after 4581 sec. Lower patterns: descending limb, photograph taken after 4731 sec.

Other Methods. Weight fractions of the protein solutions, which were required for extinction coefficient and biuret factors, were determined by drying aliquots (at approximately 75°) in tared microplatinum boats to constant weight in an evacuated (0.05 mm) pistol tube containing P_2O_5. Weighings were carried out to the nearest 0.001 mg (Ainsworth Magni-Grad type FHM, microbalance). Ash was determined on these dried samples by igniting in a double porcelain crucible over a meker burner. Aliquots of the dialysis fluids[5] were subjected to the identical treatment aud the final dried weights were determined by difference. Measurements of the ultraviolet absorption spectra (Cary 14) and biuret factor (Gornall *et al.*, 1949) were made on the same protein solutions whose weight fractions were determined. Values for extinction coefficients reported here were made in 0.05 M sodium phosphate (pH 7.00) at 25°.

Results and Discussion

Homogeneity Measurements. Electrophoresis studies on cellulose acetate at an average temperature of 10° and within the pH range of *ca.* 6–8.5 (Γ/2 = 0.05 in

[2] Gelman Manual 70176B, 1966.

[3] ADP (2.8 mM), creatine phosphate (15 mM), glucose (3.7 M), $MgSO_4$ (4.1 mM), TPN, (1.2 mM), hexokinase (20 μg/ml), glucose 6-phosphate dehydrogenase (20 μg/ml), phenazine methosulfate (0.2 mg/ml), nitrotetrazolium blue (0.2 mg/ml), and Tris (Cl^-) (0.5 M, pH 7.5).

[4] Canalco Instructions Bulletin, April 1965.

[5] Because of the tendency of the brain enzyme to crystallize and then precipitate at low ionic strengths near the apparent isoelectric point, it did not prove feasible to deionize samples by gel filtration or by dialysis *vs.* distilled water prior to determination of dry weights. The procedure employed dialysis at pH 8–9 *vs.* 5×10^{-5} M NH_4OH–0.01 M KCl to displace SO_4^{2-}, followed by exhaustive dialysis *vs.* 5×10^{-5} M NH_4OH.

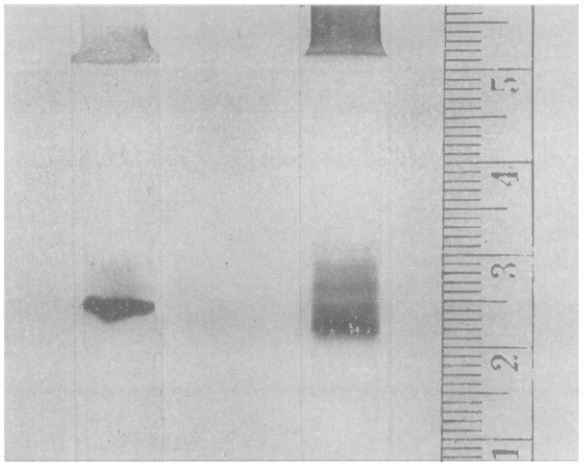

FIGURE 3: Polyacrylamide gel disc electrophoresis of crystalline calf brain ATP–creatine transphosphorylase. Left-hand photograph: results obtained with use of 7% gels photopolymerized in presence of riboflavin; right-hand photograph: results obtained with 7% gels polymerized with persulfate. Runs were conducted in a refrigerator at 4 mA/tube and at an applied voltage of initially 120 V for 60 min.

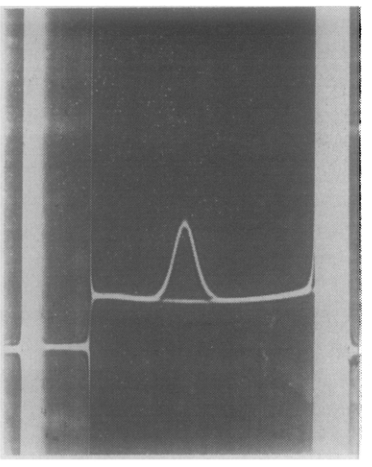

FIGURE 4: Sedimentation velocity pattern of ATP–creatine transphosphorylase from calf brain (C_0 = 7.62 mg/ml) in 0.15 M KCl, 0.01 M Tris, 0.001 M EDTA, and 0.1 M β-mercaptoethanol (pH 7.8) at 3°. Sedimentation at 59,780 rpm (in a double-sector, 12-mm Epon-filled cell); exposure taken 96 min after speed attained; schlieren diaphragm (phase-plate) angle at 70°.

0.001 M 2-mercaptoethanol) revealed only a single protein band with enzymatic activity. There appeared to be no evidence for denaturation, precipitation, or alteration of the brain enzyme molecule within this pH range. Densitometric measurements were used to determine the relative mobility in arbitrary units as shown in Figure 1, where the results, apparent mobility *vs.* pH, for the calf brain enzyme are also compared with the crystalline calf muscle isoenzyme. An extrapolated value for the apparent isoelectric point for the brain enzyme appeared to lie in the vicinity of *ca.* pH 4.5, or surprisingly acidic compared with the muscle enzyme. However, in acetate buffers below pH 6 increased aggregation, precipitation, and denaturation of the brain enzyme was evident with decreasing pH values, such that a second band of precipitated and enzymatically inactive protein remained at the origin. The region below pH 5.0 could not be penetrated because of its instability and these observations, coupled with errors in extrapolation, therefore make such a value for the isoelectric point uncertain. Above pH values of 9–9.5, instability of the brain enzyme again was evident, with a second band migrating behind the native enzyme at 9.5, and which apparently was denatured enzyme. By way of comparison, the calf muscle enzyme proved more stable and throughout the entire pH range studied only a single enzymatically active protein band was observed. Furthermore, the calf muscle enzyme shows a much smaller mobility between pH values of 6 and 9, with an apparent isoelectric point in the neighborhood of 7.8; however, because of the shallow slope in the region of 7–8.5, an error of at least 0.5 unit would be possible by this technique. In addition with the use of different lots of cellulose acetate, it became clear that some inherent error due to adsorption or retention is possible. Nevertheless, this technique, when conducted with a single lot of strips and simultaneously for both enzymes does reveal the enormous differences in the charge distribution between the two calf isoenzymes.

By liquid-boundary electrophoresis, only a single component is observable, provided measurements are within a range of pH values and ionic strengths, where the brain enzyme exhibits maximum stability, and a typical schlieren pattern is given in Figure 2. At low ionic strengths, and in the neighborhood of its apparent isoelectric point, the brain enzyme has a tendency to first crystallize and then to aggregate during dialysis; such a resulting unstable condition made it difficult to extend the measurements into the isoelectric state.

Polyacrylamide gel discontinuous electrophoresis, which made use of the Kohlrausch effect (with a pH gradient), yielded ambiguous and erratic results, usually with a multibanded pattern, when 7% gels were polymerized with persulfate (Figure 3). Attempts at removal of excess persulfate by use of thioglycolate or mercaptoethanol were only partially successful, but if the use of persulfate were avoided entirely (as had been recommended by Brewer, 1967) and the gel were photopolymerized in the presence of riboflavin, electrophoresis of the calf brain enzyme yielded essentially single-banded patterns with enzymatic activity (Figure 3). Sometimes a vague diffuse band appeared above it, and which is apparently the result of some instability at the "running pH" of 9.5 (Ornstein, 1964). Thus, the difficulties with oxidants are apparent and the contrast between the results obtained with the two types of polymerized gels is very striking (Figure 3).

By sedimentation velocity, again in presence of mercaptoethanol at pH 7.8, a single sedimenting component is observed (Figure 4) with no evidence of heterogeneity, aggregation, or dissociation throughout the entire period of sedimentation.

Thus, it may be taken as demonstrated, that under conditions where the native molecule is apparently stable, it satisfied criteria of purity by several electrophoretic and sedimentation techniques which include sedimentation equilibria. However, because the use of reducing agent apparently is necessary to stabilize the

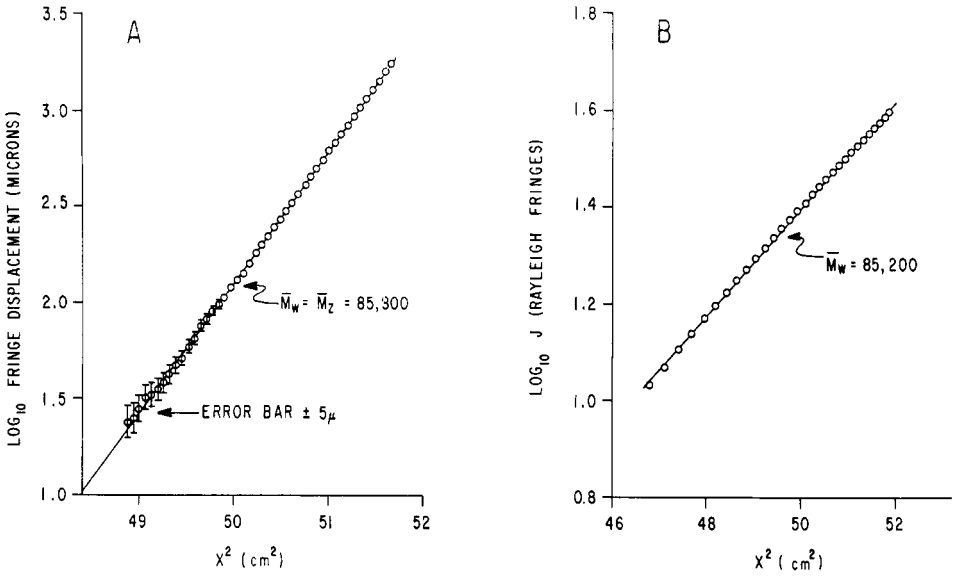

FIGURE 5: Sedimentation equilibrium measurements at 3° of ATP–creatine transphosphorylase from calf brain; in 0.15 M KCl, 0.01 M Tris, 0.001 M EDTA, and 0.1 M β-mercaptoethanol (pH 7.8). (A) A molecular weight determination by the meniscus depletion technique of Yphantis (1964), expressed in terms of log fringe displacement in microns (blank corrected) *vs.* X^2 (square of the distance, in cm^2, from the axis of rotation). C_0 = 0.20 mg/ml and the time for the data given here was 24 hr at 17,200 rpm. The indicated ranges in the plot correspond to ±5-μ fringe displacement; only points with a net displacement greater than 100 μ have been used for calculations (see Yphantis, 1964). (B) A molecular weight determination from a low-speed 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 cm^2, from the axis of rotation). The initial concentration of protein was 5.3 mg/ml. The protein was sedimented initially at 11,250 rpm for 5 hr and then the speed reduced to 6995 rpm. The time for the data given here was 92 hr after equilibrium speed (6995 rpm) was reached at 3°, and no significant differences were noticed between 52 and 102 hr. For both A and B, $\overline{M}_w$ is calculated from $\overline{M}_w(1 - \overline{V}\rho)$ at 3°, with a $\overline{V}^\circ_{3^\circ}$ $(c \rightarrow 0)$ = 0.736 (extrapolated from data of Noda *et al.* (1954) for the rabbit muscle enzyme) and a measured ρ of 1.009136.

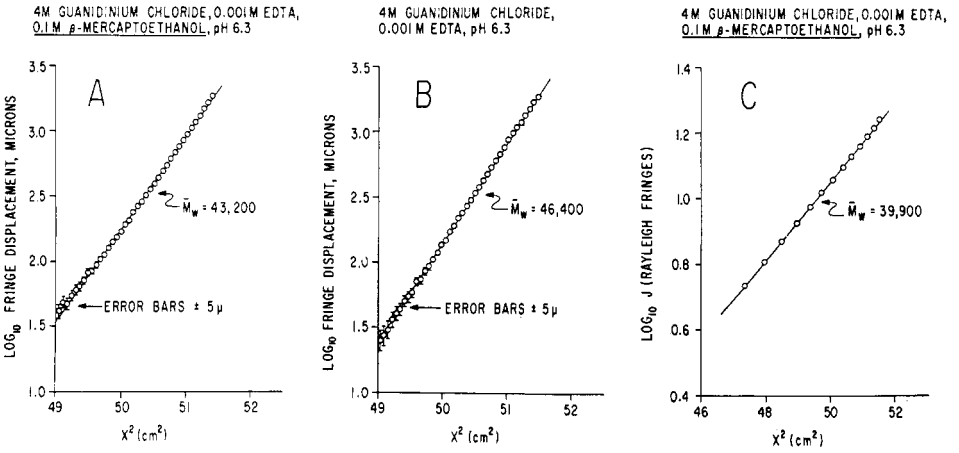

FIGURE 6: Analyses of the polypeptide chains in ATP–creatine transphosphorylase from calf brain by sedimentation equilibrium experiments at 20° in 4 M guanidinium chloride solutions, with and without 2-mercaptoethanol. (A) High-speed molecular weight determination by the procedure of Yphantis (1964), expressed in terms of logarithm of the fringe displacement in microns (blank corrected) *vs.* X^2 (square of the distance, in cm^2, from the axis of rotation) for 0.41 mg/ml of protein (initial) in 4 M guanidinum chloride, 0.001 M EDTA, and 0.1 M β-mercaptoethanol (pH 6.3). The time for data given here was 28 hr at 29,500 rpm, 20°. (B) High-speed molecular weight determination by procedure of Yphantis (1964), for 0.2 mg/ml of protein (initial) in 4 M guanidinium chloride–0.001 M EDTA (pH 6.3), 20°, and in the *absence* of mercaptoethanol. The time for the data given here was 28 hr at 29,000 rpm. Evidence for slight aggregation at the bottom of the cell, if times were extended much beyond this point. (C) Low-speed molecular weight determination (Richards and Schachman, 1959; Yue *et al.*, 1967a,b), expressed in terms of log (log of the protein concentration in Rayleigh fringes) *vs.* X^2 (square of the distance in cm^2, from the axis of rotation) for 2.9 mg/ml of protein (initial) in 4 M guanidinium chloride, 0.001 M EDTA, and 0.1 M β-mercaptoethanol (pH 6.3) at 20°. The protein was sedimented initially at 23,150 rpm for 2.5 hr and the speed was reduced to 12,590 rpm. The time for the data given here was 50 hr after equilibrium speed (12,590 rpm) was reached at 20°, and no significant differences were noticed between 22 and 50 hr.

TABLE I: Physical Properties of ATP–Creatine Transphosphorylase from Calf Brain and Its Component Polypeptide Chains.

A. Kinetic Unit (Native Enzyme)	
pI_0, isoelectric point ($\Gamma/2 \rightarrow 0$)	5.6
$s^0_{3°,b}$, sedimentation coefficient with 2-mercaptoethanol ($\bar{c} \rightarrow 0$, pH 7.81), sec	3.32×10^{-13}
$s^0_{20,w}$, sedimentation coefficient[a] ($\bar{c} \rightarrow 0$, pH 7.8), sec	5.42×10^{-13}
$D^0_{20,w}$, diffusion coefficient (cm²/sec) ($c \rightarrow 0$, pH 7.8) calculated from $s_{20°,w}$ and M_{eq}	6.02×10^{-7} ($D^0_{3°,b} = 3.48 \times 10^{-7}$)
M_{equil}, (molecular weight by sedimentation[b] equilibrium, pH 7.8), (g/mole)	85,200
f/f_0, frictional ratio (and calculated axial ratio, a/b for assumed anhydrous prolate ellipsoid of revolution)	1.21 ($a/b = 4.4$)
$(\Delta n/\Delta c)_{3°}$, refractive index increment[c] ($\lambda_{546\ m\mu}$ (g/100 ml)$^{-1}$	(1.98×10^{-3})
Biuret factor (λ_{540} mμ, 10-ml volume, 1-cm light path) (mg/absorbance unit)	34.0
$E^{1\%}_{1\ cm}$, extinction coefficient (280 mμ, pH 7.00, 0.05 M sodium phosphate)	8.24
Ratio of extinction coefficients at 280/260 mμ	1.77
Ratio of extinction coefficients at 280/259 mμ	1.78
B. Polypeptide chains (noncovalently linked)	
$\bar{M}_w$, weight-average molecular weight[d] (2.9 mg/ml, 0.001 M EDTA–4 M guanidine hydrochloride–0.01 M 2-mercaptoethanol, pH 6.3)	39,900
$\bar{M}_w$ (0.22 mg/ml, 0.001 M EDTA, 4 M guanidine hydrochloride, and 0.10 M 2-mercaptoethanol, pH 6.3)	42,200
$\bar{M}_w$ (0.41 mg/ml, 0.010 M EDTA, 4 M guanidine hydrochloride, and 0.1 M 2-mercaptoethanol, pH 6.3)	43,200
$\bar{M}_w$ (0.22 mg/ml, 0.001 M EDTA, 4 M guanidine hydrochloride, and *without* 2-mercaptoethanol, pH 6.3)	46,400

[a] Calculated to reference state at 20° with the use of a $\bar{V}^0_{3°}$ ($c \rightarrow 0$) = 0.736 and $\bar{V}^0_{20°}$ ($c \rightarrow 0$) = 0.744 (extrapolated data from Noda *et al.* (1954) for rabbit muscle enzyme). [b] Calculated from $\bar{M}_w(1 - \bar{V}\rho)$ at 3° with a $\bar{V}^0_{3°}$ ($c \rightarrow 0$) = 0.736 (Noda *et al.*, 1954) for rabbit muscle enzyme and a ρ = 1.009136 g/cm. [c] Estimated as described by Yue *et al.* (1967a), from differential Rayleigh fringes, J, measured with a synthetic boundary cell and Model E ultracentrifuge as a Rayleigh interferometer. [d] Calculated from $\bar{M}_w(1 - \bar{V}\rho)$ with use of a $\bar{V}$ = 0.735 cm³/g (Noltmann *et al.* (1962) calculated from the amino acid composition of rabbit muscle enzyme (see Yue *et al.*, 1967b)) and a ρ = 1.0973 at 20°.

enzyme during the time required for these measurements, some accuracy may be sacrificed, than when these same physical methods may be applied without the introduction of an additional component to the system.

Sedimentation Velocity and Sedimentation Equilibrium Studies on the Native Enzyme. At 0.16 ionic strength, pH 7.8 and in the presence of 0.1 M mercaptoethanol (under which conditions the enzyme appears to sediment as a single component), the observed sedimentation coefficients (at 3° and buffer) over the relatively narrow protein concentrations explored ($C_0 \cong$ 2–7.6 mg/ml) appear to decrease very slightly with an increase in protein concentration according to the expression: $s'_{3°,b} = 3.32 \cdot (1\text{–}0.011\bar{c}) \times 10^{-13}$ sec, where $\bar{c}$ is expressed in milligrams per millititer. Although this small concentration dependency and slight negative slope might be interpreted as characteristic of a compact globular macromolecule, nevertheless, one should bear in mind that this slight negative slope, over the narrow range concentration employed could not preclude weak interactions, nor was the concentration range wide enough to reveal any effect of association–dissociation equilibria in presence of 0.1 M mercaptoethanol (Nichol *et al.*, 1964). It is interesting that the calculated value of $s^0_{20,w}$ = 5.42 S (extrapolated to zero protein concentration) is only just slightly higher than the value for the rabbit muscle enzyme reported before (Yue *et al.*, 1967b) at 7.8 and $\Gamma/2 \cong 0.16$, *viz.*, 5.31 S. Moreover, the values for the frictional ratio, f/f_0, and for the calculated axial ratio, a/b, appear nearly identical for both rabbit muscle and calf brain enzyme and which might imply similar over-all gross molecular shapes, in solution.

Sedimentation equilibrium studies on the native calf brain enzyme, by both the high-speed meniscus-depletion technique of Yphantis (1964) and by the classical low-speed technique (Figure 5), in the presence of identical solvent conditions as employed for the velocity runs, yield values for $\bar{M}_w \cong$ 85,000 (with essentially identical values (Figure 5A) for the weight averages, *i. e.*, $\bar{M}_w = \bar{M}_z$). A value of 85,200 from the more reliable low-speed analyses (Figure 5B) is taken as the best preliminary estimate for its molecular weight, and which

value may hinge to some degree on the value of $\bar{V}$ selected (see legend, Figure 5); an estimate of $\bar{V}$ from its amino acid composition (in progress) may alter this value of the molecular weight slightly. With this reservation, it is nevertheless of interest that its molecular weight appears to be just slightly higher than the value assigned to the rabbit muscle enzyme, *viz.*, 82,600 (Yue *et al.*, 1967b), and as will be shown in a future communication also just slightly higher than that of the native calf muscle isoenzyme, *ca.* 83,000 (Jacobs *et al.*, 1968). It would be tempting, therefore, to point out the possibility that the component polypeptide chains (see below) of the brain enzyme may be slightly heavier than those of its muscle enzyme counterpart, but with the reservation that $\bar{V}$ may not alter significantly.

Sedimentation Equilibrium Studies on the Polypeptide Chains in 4 M *Guanidinum Chloride.* Based on the extensive studies on the rabbit muscle enzyme in several dispersing solvents and at varying concentrations of these denaturants (Yue *et al.*, 1967b), comparable sedimentation equilibrium analyses were conducted in the most efficient of these dispersing solvents (*i.e.*, 4 M guanidinum chloride containing 0.001 M EDTA) *with* and *without* 0.1 M β-mercaptoethanol (Figure 6A, B), and by both high- and low-speed sedimentation equilibrium techniques (*cf.* Figure 6A,C), to reveal any effects of nonideality. It is apparent that a weight-average molecular weight of approximately one-half that of native enzyme results and that the value is not too much altered even in the absence (Figure 6B) of mercaptoethanol (although aggregation slowly affects the measurements and makes such measurements in the absence of mercaptoethanol difficult). Therefore interchain disulfide bridges between the two polypeptide chains seem to be excluded. Suitable extrapolations, as that employed for the rabbit muscle enzyme (Yue *et al.*, 1967b) to eliminate or minimize problems of preferential binding, nonidealty, or effect on $\bar{V}$, have not as yet been undertaken, but if the analogy to the rabbit muscle enzyme holds, measurements by the Yphantis technique at protein concentrations less than 0.2 mg/ml are not likely to be in error by more than 4% (high), from the hypothetical value of the half-kinetic unit. It is likely, however, that some nonideality effect is present, *cf.* $\bar{M}_w$ values of 39,900 *vs.* 43,200 at pH 6.3 for initial protein concentrations of 2.9 and 0.41 mg per ml, as measured by the low- and high-speed technique, respectively (Figure 6A,C), and 42,200 at *ca.* C_0 = 0.22 mg per ml by the Yphantis technique (not shown) and with no unusual discrepancies in their weight averages. For the present, in the absence of any other supporting chemical data, tentatively, it appears that the calf brain enzyme like the rabbit muscle enzyme (Yue *et al.*, 1967b) is composed of two noncovalently linked polypeptide chains, with similar, if not identical, molecular properties.

A summary of all the physical properties measured for the calf brain enzyme and pertinent to this report is given in Table I. These data also include: an estimated value for its isoelectric point at zero ionic strength (see below), a calculated value for its translational diffusion coefficient, a measured value at 280 mμ for its ultraviolet extinction coefficient at its absorption maximum, its

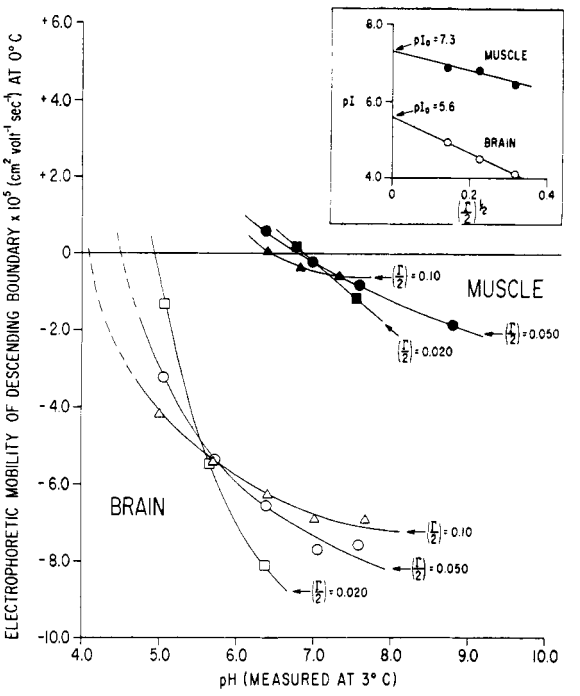

FIGURE 7: Electrophoretic mobility for 0° of ATP–creatine transphosphorylase from calf brain and calf muscle as a function of pH (measured at 3°) at several ionic strengths. Lower curves: open symbols for calf brain ATP–creatine transphosphorylase (all buffers contained 0.010 M β-mercaptoethanol): (△——△) 0.10 ionic strength buffers containing 0.05 ionic strength KCl; buffer Tris–KCl–HCl, imidazole–KCl–HCl, and potassium acetate–KCl–acetic acid at pH 7.67, 7.02, 6.41, 5.71, and 5.02, respectively. (○——○) 0.050 ionic strength buffers, buffer: Tris–HCl, imidazole–HCl, and potassium acetate–acetic acid at pH 7.58, 7.05, 6.39, 5.72, and 5.06, respectively. (□——□) 0.020 ionic strength buffers; buffer: imidazole–HCl, potassium acetate–acetic acid at pH 6.37, 5.65, and 5.07, respectively. Upper curves: closed symbols for calf muscle ATP–creatine transphosphorylase (no mercaptoethanol added to the buffers): (▲——▲) 0.10 ionic strength buffers containing 0.05 ionic strength KCl; buffer: Tris–KCl–HCl and imidazole–KCl–HCl at pH 7.33, 6.82, and 6.41, respectively. (●——●) 0.050 ionic strength buffers containing 0.00–0.025 ionic strength KCl; buffer: Tris-KCl-HCl and imidazole–KCl–HCl at pH 8.80, 7.59, 6.99, and 6.37, respectively. (■——■) 0.020 ionic strength buffers; buffer: imidazole–HCl at pH 7.55 and 6.78. Upper insert: p*I*, isoelectric point, *vs.* $(\Gamma/2)^{1/2}$, square root of ionic strength. (●——●) for calf muscle enzyme, pI_0 = 7.3; (○——○) for calf brain enzyme, pI_0 = 5.6.

biuret factor, an estimate of its refractive index increment, and a calculated value for its molar frictional ratio. With the use of Perrin's (1936) equation an f/f_0 value of 1.21 would correspond to an axial ratio of only 4.4 for the major to minor axes, a/b, of an assumed anhydrous prolate ellipsoid of revolution; this value proves to be identical with that of the rabbit muscle enzyme (Yue *et al.*, 1967b), and one might infer that the two enzymes are not too dissimilar in their over-all gross physical shape. Finally, the data obtained on the subunit weight-average molecular weights are summarized (measured with and without 2-mercaptoethanol) in support of the tentative conclusion that, like the rabbit muscle enzyme (Yue *et al.*, 1967b), the calf

brain enzyme also consists of two polypeptide chains, noncovalently linked.

Liquid-Boundary Electrophoresis Studies. Examination of the electrophoretic behavior of the native calf brain protein had revealed that the enzyme migrated in an electrical field as a single component, provided that measurements were made within the pH stability range of the molecule (*e.g.*, Figure 2), and that data at acid pH values in the neighborhood of its isoelectric point and at low ionic strengths could not be extended beyond the range given in Figure 7 because of the instability of, and resulting aggregation of the protein at 0° (even in the presence of mercaptoethanol). Therefore, there is some uncertainty in the extrapolation of the mobility data to a pH value at zero mobility and measurements are highly uncertain at pH values less than 6. Nevertheless, it is evident that the isoelectric state (defined as the point of zero mobility) is dependent to some degree upon the ionic strength, although its dependency is far less than that which had been observed for the yeast nucleoside triphosphate–nucleoside diphosphate transphosphorylase (Yue *et al.*, 1967c). The linear extrapolation permitted by the pH *vs.* $(\Gamma/2)^{1/2}$ plot (Figure 7) leads to a value of $pI_0 = 5.6$ (at zero ionic strength). When the data for the calf brain enzyme are compared with the data obtained for the calf muscle isoenzyme (Figure 7), it is clear that the absolute values for electrophoretic mobility of the muscle enzyme, at any given pH value (between 6.5 and 8) are far smaller than those for the brain enzyme and qualitatively they confirm the cellulose acetate measurements. The calf muscle enzyme appeared to migrate as a single component over the range of conditions studied and instability problems were not encountered between pH values *ca.* 6.4 and 8.8. Also, extrapolation to zero ionic strength leads to a pI_0 of 7.3 for the calf muscle enzyme, and somewhat more acidic than estimated by runs on cellulose acetate, which appeared to be subject to small retention and adsorption effects. Thus, it is clear that the two calf isoenzymes, which catalyze similar reactions, must differ enormously in their gross electrical properties in solution. Whether their charge distributions in the microregion of their active sites are identical, however, or whether their overall charge distributions may affect their kinetic properties, are problems which may possibly be solved by a careful comparison of their kinetic parameters on the native molecule and on suitable and active protein derivatives. It will be of further interest to compare their theoretical titration curves deduced from their respective amino acid analyses (as calculated previously for the rabbit muscle enzyme; Noltmann *et al.*, 1962) with their electrophoretic behavior and with these estimates for their pI_0 values.

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