Studies on Adenosine Triphosphate Transphosphorylases. VII. Isolation of the Crystalline Adenosine Triphosphate–Creatine Transphosphorylase from Calf Brain*

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ABSTRACT: A procedure is described for the isolation of crystalline adenosine triphosphate–creatine transphosphorylase in good yield, from calf brain extracts. Some observations on the stability properties of the enzyme are presented with respect to pH, temperature, and reducing agents, to provide a basis for a systematic physical–chemical and kinetic characterization.

With the isolation of this enzyme from calf brain, a comparison is now possible with its crystalline isoenzyme counterpart from calf muscle (Jacobs, H. K. Keutel, H. J., Yue, R. H., Okabe, K., and Kuby, S. A (1968), *Federation Proc. 27*, 640) as well as with crystalline enzymes that were isolated from the brain and skeletal muscle of man (Jacobs, H. K., Keutel, H. J., Yue, R. H., Okabe, K., and Kuby, S. A. (1968), *Federation Proc. 27*, 640) and of rabbit (Kuby, S. A., Noda, L., and Lardy, H. A. (1954), *J. Biol. Chem. 209*, 291).

Adenosine triphosphate–creatine transphosphorylase is presumed to play an important role in the metabolism of cerebral tissues (*e.g.*, McIlwain, 1966; Nachmansohn and Wilson, 1951) in a manner analogous to its now accepted physiological and catalytic role in muscle tissues (*e.g.*, Davies *et al.*, 1967; Mommaerts and Wallner, 1967). Thus, it is presumed that it provides a means for the resynthesis of ATP *via* creatine phosphate through ADP, and it should not be forgotten that this concept had its origins in the pioneer work of Meyerhof and associates (*e.g.*, Meyerhof and Lohmann, 1932).

Relatively few enzymes have been isolated and obtained in crystalline form from extracts of mammalian cerebral tissues, where the recognized problems of phospholipids often present obstacles to the isolation of proteins in reasonable yield and purity and also in a reproducible manner. (But a preliminary report of one case has recently appeared, *viz.*, that of the crystalline triose phosphate dehydrogenase from rabbit brain; Lebherz and Rutter, 1967.) Efforts to purify the ATP–creatine transphosphorylase from calf brain extracts were reported early (Kuby and Mahowald, 1958), and the initial steps in the purification scheme to be reported here were retained from the earlier procedure. More recently, other attempts to isolate this enzyme from cerebral tissues have been reported and a highly purified preparation was obtained, *e.g.*, from ox brain (Wood, 1963a,b); and Eppenberger *et al.* (1967) have attempted to isolate a brain-type enzyme from chicken heart, which they have stated to be identical with the chicken brain enzyme itself.

Since studies from this laboratory have dealt with the general problem of ATP transphosphorylation, and, in particular, with three types of ATP transphosphorylases which had been isolated in crystalline form (the ATP–creatine transphosphorylase from rabbit muscle (Kuby *et al.*, 1954), the ATP–AMP transphosphorylase from rabbit muscle (Noda and Kuby, 1957), and the nucleoside diphosphokinase from brewers' yeast (Ratliff *et al.*, 1964)), and since the rabbit muscle ATP–creatine transphosphorylase has been the subject of extensive investigation (*e.g.*, see Kuby and Noltmann, 1962), it was of interest to broaden the physicochemical comparison to include the isoenzymes of the ATP–creatine transphosphorylase from several mammalian species. It was hoped, thereby, to reveal from such comparative studies those similarities and differences which may be ultimately correlated with, and applied to a more intimate understanding of its mechanism(s) of catalysis. Future reports will deal with studies on the crystalline ATP-creatine transphosphorylases isolated from the muscle and brain of the calf, human, and rabbit. The enzymes isolated from human tissues will likely be of interest to those concerned with the problem of progressive muscular dystrophy where the appearance of the enzymatic activity in the serum seems to reflect the disorder (Tyler, 1966).

This report will deal specifically with the isolation of the crystalline calf brain enzyme and a brief description of its stability properties. Reports will follow which will deal (a) with a homogeneity analysis by several techniques; with its molecular weight and subunit structure, as deduced physically (Yue *et al.*, 1968); (b) with an analysis of the reactive SH groups of the native molecule and the total SH content, as also related to the stability and to the molecular structure; and finally, (c) with a kinetic analysis of its catalyzed reaction which may have a bearing on its native molecular structure

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and mechanism of action. Additional chemical studies on the protein in particular on its amino acid composition and terminal groups, will also be the subject of a later communication. A preliminary report of some of these investigations has been given (Jacobs *et al.*, 1968).

Experimental Procedure

Materials and Methods. Calf brains were removed from the animals immediately after slaughter and transported in ice to the laboratory. The hemispheres were excised, dura and external blood vessels were removed, washed (ice water) free of external blood clots, sealed in cellophane bags, and frozen at −15°. The frozen tissue (hemispheres), which was stored for periods of 6–50 days at −15°, was the source for isolation of the enzyme.

$(NH_4)_2SO_4$ was obtained from Mann (Ultra Pure) or from Mallinckrodt (A. R., recrystallized from aqueous ethanol), disodium EDTA from Fisher Scientific Co.; Tris, and the sodium salts of ATP, of creatine phosphate, and of ADP from Sigma. β-Mercaptoethanol (Eastman Chemical) was redistilled under vacuum; 95% ethanol was redistilled as described (Ratliff *et al.*, 1964). The following chemicals were obtained from the sources indicated: creatine (Eastman Chemicals' White Label), glycine (Matheson Coleman and Bell), crystalline bovine plasma albumin (Armour), and succinic acid (Mallinckrodt, A. R.). All other reagents were of analytical grade. Twice-distilled deionized water was used for preparation of all reagents including the dialysis fluids; in some cases, solutions were prepared in degassed (by boiling), distilled water or saturated with nitrogen. Lots of DEAE-cellulose (Bio-Rad Cellex-D anion exchanger, capacity *ca.* 0.66 mequiv/g) were freed of fines by decantation, cycled through 0.2 N NaOH–H_2O–0.2 N HCl–H_2O–0.2 N NaOH–H_2O as described (Ratliff *et al.*, 1964), and stored as a moist filter cake (averaging 30% dry weight) in sealed containers. Phosphocellulose (Bio-Rad Cellex-P cation exchanger, capacity 0.88–1.06 mequiv/g) was freed of fines, converted into the sodium phase with a 20-fold excess of 0.5 M NaCl in 0.5 M NaOH, washed until neutrality, and transferred into the H^+ phase by titration (ice bath) with 1.0 N HCl to pH 2.5; after washing, by filtration, with 0.01 N HCl (in the cold) followed by water until free of Cl^-, it was also stored as a moist filter cake, whose dry weight was determined. Sephadex G-75 and G-100 (bead form) were products of Pharmacia and were swelled at 3° for several days in a solution of 30% saturated $(NH_4)_2SO_4$ (1.17 M) containing 0.01 M EDTA (pH 7.4) prior to preparation of the column.

Measurements of Enzymatic Activity. To permit a comparison of the specific activity of this preparation from calf brain with that of the rabbit muscle, the original colorimetric procedure for creatine phosphate and reaction mixture conditions of Kuby *et al.* (1954) were employed during the isolation procedure with only slight modifications in the dilutions of the enzyme. Dilutions, at ice-bath temperature, were made in 1×10^{-3} M glycine (pH 9.0) (5°), in 1×10^{-3} M cysteine–1×10^{-3} M EDTA (pH 7.0) (5°), or in 0.1 M β-mercaptoethanol–1×10^{-3} M EDTA (pH 7.4) at the terminal stages of isolation. Microaliquots (5 or 10 μl) of properly diluted enzyme were pipetted directly into 10 ml of reaction mixture to minimize inactivation at high dilutions, and bovine serum albumin (1 mg/ml) was added to the glycine or cysteine solutions, if dilutions were greater than 1×10^6. The definition of a unit of activity under these conditions was as described originally (Kuby *et al.*, 1954) in terms of an apparent second-order velocity constant, k' (1 unit = k' = 1 ml μmole^{-1} min^{-1}).

In addition, the titrimetric pH-Stat method previously described (Mahowald *et al.*, 1962) was utilized to follow the purification, but with a reaction mixture which consisted of: 0.04 M creatine, 0.004 M ATP, 0.004 M $MgSO_4$, and 1 mg/ml of albumin, 30°, pH 8.8; with 0.010 N NaOH as titrant (see also Specifications and Criteria, 1967), and with an 8.0-ml reaction volume under a nitrogen barrier. The definition of a unit of activity for this case was in terms of 1 unit equal to 1 μequiv/min, as measured from the initial velocity. To interconvert from one set of conditions to the other, it was found that the pH-Stat-defined unit (1 μequiv/min) was approximately 1.8–2 times that determined under colorimetric conditions (and defined with a k' = 1 ml μmole^{-1} min^{-1}).

Protein was determined by the colorimetric biuret procedure of Gornall *et al.* (1949) or in later stages by its extinction coefficient at 280 mμ. Appropriate biuret factors and extinction coefficients for some fractions are presented in the text and these values for the isolated enzyme will be presented (Yue *et al.*, 1968). Specific activity is expressed in terms of units per milligram of protein.

Isolation Procedure (from Calf Brain). All steps were carried out in a cold room (2–4°) or in an ice bath, unless otherwise specified (*e.g.*, −10° for the solvent steps); pH measurements were made at or near 5° with a Radiometer TTTla meter, equipped with scale-expander PHA 630Ta, and A. H. Thomas glass-calomel electrodes. The required amounts of solvents and $(NH_4)_2SO_4$, as well as the concentrations of these reagents in the precipitated pellets, were estimated by the formulas summarized by Noltmanm *et al.* (1961), and a saturated solution of $(NH_4)_2SO_4$ was taken to be 3.9 M at 0°.

FRACTION I. The frozen brain tissue (*ca.* 6 kg) is thawed overnight (at 2–5°), passed through a meat grinder, and collected in a tared polyethylene tray. The tissue is then blendorized for 30 sec (gallon-size Waring Blendor) in chilled 0.1 M ammonium acetate (pH 9.5), with use of 2.0 l./kg of ground tissue, and the homogenate was stirred slowly in the cold room for 24 hr.

FRACTION II. The suspension without centrifugation is transferred to a −10° bath and chilled to 0°, with stirring. A volume of 95% ethanol (−10°) equal to the total homogenate volume is added slowly with good mechanical stirring (the temperature should not rise above 3°). After the ethanol addition is completed and now presumed to be at 50% (v/v) of 95% ETOH, the temperature of the mixture is allowed to adjust, with stirring, to −8 to −10°. The mixture is centrifuged at 1300*g* for 45 min at −10°, the supernatant liquid in-

cluding loosely packed material is decanted, and its volume is determined. The solution (−10°) is adjusted to a final concentration of 0.015 M $MgSO_4$ by the slow addition, with stirring, of a calculated volume of 2.0 M $MgSO_4$ (pH 8.7) (measured at 25°), and the ethanol concentration is increased to 70% (v/v of 95%). The suspension is centrifuged at 1300*g* for 45 min, at 10°, and the tightly packed reddish pellet is suspended rapidly at 0° in a volume of 0.001 M NH_4OH equal to $^1/_{20}$ of the calculated supernatant liquid volume of fraction I[1] and dialyzed with continuous flow (Noltmann *et al.*, 1961) against 50 l. of 0.001 M NH_4OH overnight. After dialysis, the insoluble material is removed by centrifugation at 15,000*g* for 60 min. The protein concentration of the supernatant liquid (fraction II) is determined (after precipitation with 10% trichloroacetic acid, followed by the biuret procedure (Gornall *et al.*, 1949) with a factor of 32.5 mg/10-ml volume per unit of absorbance) and the protein concentration is adjusted, if possible, to 8.0 mg/ml with the dialysis fluid; otherwise, to 6 mg/ml.

FRACTION III. For an initial protein concentration of 8 mg/ml, fractionation between 0.43 and 0.64 $(NH_4)_2SO_4$ saturation is employed; but for the case of 6 mg/ml, 0.43–0.67 saturation is used. In an ice bath with stirring, solid $(NH_4)_2SO_4$ is slowly added and the pH is simultaneously maintained at 7.0 by the addition of either 1 M NH_4OH or 1 N H_2SO_4. After the $(NH_4)_2SO_4$ concentration has been adjusted to 0.43 saturation, it is allowed to stir slowly for 2 hr, followed by centrifugation at 15,000*g* for 45 min. The $(NH_4)_2SO_4$ concentration of the supernatant liquid is increased to 0.64 saturation (or 0.67) by the addition of solid $(NH_4)_2SO_4$, at pH 7.0; and after a 2-hr stirring period, the precipitated protein is centrifuged and dissolved in a volume of 0.001 M EDTA (pH 8.0) equal to $^1/_{70}$ the volume of fraction II. It is then dialyzed by continuous flow against 50 l. of 0.001 M EDTA (pH 8.0) overnight at which time the dialysis fluid should be free of SO_4^{2-}. The protein solution is clarified by centrifugation and is designated fraction III, whose protein concentration and volume are determined.

FRACTION IV. During the dialysis period, the phosphocellulose is equilibrated as follows. A calculated amount of the moist cake is suspended in 0.001 M EDTA (Na^+) (pH 6.8) such that the final concentration (in dry weight) is 30 mg/ml and the total amount will be equal to at least 30 times the protein concentration of fraction III. The suspension is titrated with stirring to pH 6.8 (with 1 N NaOH) at 5°, transferred quantitatively to a sintered-glass funnel, washed with 0.001 M EDTA equal to four times the volume initially employed, resuspended in 0.001 M EDTA, and the pH is readjusted, if necessary. The following day the procedure is repeated until the pH is stable at 6.8, and a calculated volume of the phosphocellulose containing 30 times the protein weight of fraction III, is then deaerated by suction, transferred to a 5.0 × 50 cm column, packed under a few pounds of N_2 pressure and finally with a peristaltic pump (Harvard Model 600–1200) at a flow rate of *ca.* 100 ml/hr, to yield a bed height of *ca.* 30 cm when tightly packed. Fraction III is adjusted to pH 6.8 (with 0.1 N NaOH or 0.01 N HCl) and applied to the phosphocellulose column under N_2 pressure (*ca.* 5–6 lbs). The single unretarded peak (containing the enzymatic activity), which is monitored at 280 mμ (Vanguard 1056 or GME, and a Texas instrument recorder), is displaced off the column at *ca.* 100 ml/hr with the same solvent (0.001 M EDTA, pH 6.8). A large reddish-brown band (hemoglobin and derivatives) is retained by the column. The protein peak is collected, adjusted to 0.85 saturation $(NH_4)_2SO_4$, at pH 7.0, and after a 2-hr period (with slow stirring) the precipitate is collected by centrifugation at 15,000*g* for 75 min. The pellet is resuspended (in one-fifth volume of fraction III) in a solution of 0.005 M succinate (0.0115 M Tris)–0.001 M EDTA–0.001 M β-mercaptoethanol (pH 7.5) and dialyzed by continuous flow *vs.* 50 l. of the same solution overnight. After the SO_4^{2-} concentration of the dialysis fluid has been reduced to a negligible concentration, the protein solution is clarified by centrifugation (25,000*g*, 60 min) and the volume of fraction IV and its protein concentration is determined.

FRACTION V. An amount of DEAE-cellulose equal to at least 20 times the total protein of fraction III is suspended in 0.005 M succinate (0.0114 M Tris)–0.001 M EDTA (pH 7.5) at a concentration of *ca.* 30 mg (dry weight)/ml; its pH is readjusted and allowed to equilibrate overnight. It is then washed (by filtration) with four times the original volume of buffer originally used, resuspended in the same buffer, and an amount equal to 25–35 times the protein of fraction IV is transferred to a 2 × 150 cm column. It is now further equilibrated by pumping two bed volumes (at *ca.* 100 ml/hr) of the same buffer which now contains 0.001 M β-mercaptoethanol. Fraction IV is applied to the column and after passage of one bed volume (at 1.7 ml/min), a linear gradient with respect to succinate ion is initiated by the use of two cylinders, each containing 1.5 l. of buffer: the mixing chamber (8.9-cm i.d. × 48.4 cm) containing 0.005 M succinate (0.0115 M Tris)–0.001 M EDTA–0.001 M β-mercaptoethanol (pH 7.5) and the reservoir chamber, 0.09 M succinate (0.186 M Tris)–0.001 M EDTA–0.001 M β-mercaptoethanol (pH 7.5). The chromatogram is monitored at 280 mμ and analyzed for enzymatic activity (see Figure 1). A complex chromatogram is evident, and the enzyme appears beneath a skewed protein peak between conductance values of *ca.* 1.9 and 3.8 mmhos (as measured, *e.g.*, with a Radiometer conductivity meter DCM-2d) but with no evidence at this point of multiple enzymatic species. The fractions containing enzymatic activity are pooled and its final protein concentration is estimated at 280 mμ, assuming that 1 mg/ml has an OD_{280} of 0.805 at this point. The protein is concentrated as follows, by adsorption on and elution from DEAE-cellulose. The succinate concentration of the protein solution is diluted to a final concentration of 0.015 M succinate (checked conductometrically) by the addition of 0.001 M EDTA (pH 7.5). An aliquot of DEAE suspension, equilibrated as above and equivalent

[1] Determined by centrifuging an aliquot at 100,000*g* for 6 min.

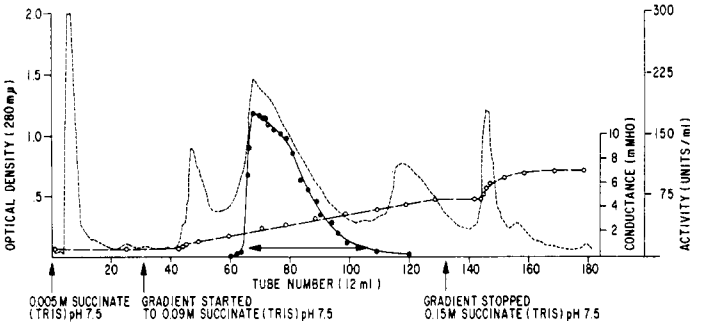

FIGURE 1: Chromatography of fraction IV (see text) of ATP–creatine transphosphorylase from calf brain on DEAE-cellulose 2.0 × 150 cm, 1.7 ml/min. The developing systems and the use of a linear gradient in succinate ion concentration are described in the text and pertinent information is presented in the above figure. (- - - -) Optical density recorded at 280 mμ; (●——●) enzymatic activity in units per milliliter (1 unit = k' = 1; see text); (○——○) conductance in millimhos.

to 40 times (dry weight) of the protein, is filtered on a coarse, sintered-glass filter; and the filter pad is transferred to the protein solution which is then suspended by stirring, with the pH maintained at 7.5. After 30 min, the DEAE-cellulose is transferred quantitatively to the same funnel by the addition of a small volume of 0.005 M succinate–0.001 M EDTA–0.001 M mercaptoethanol (pH 7.5); and the enzyme is eluted batchwise by the addition of 150 ml of 0.2 M succinate (~0.425 M Tris)–0.001 M EDTA–0.001 M mercaptoethanol (pH 7.5) divided into four volumes. To the combined filtrates, solid $(NH_4)_2SO_4$ is added to 0.90 saturation at pH 7.5; after at least 2 hr, with slow stirring, the precipitate is collected by centrifugation (25,000*g*) and the pellet is dissolved in 0.001 M EDTA (Na^+)–0.01 M mercaptoethanol (pH 7.5) to yield a final protein concentration of *ca.* 25 mg/ml.

FRACTION VI. The $(NH_4)_2SO_4$ concentration of fraction V is estimated from the volume increase of the dissolved pellet (*i.e.*, $S'' = \Delta V(S')/V''$; Noltmann *et al.*, 1961) and increased to 0.40 saturation at pH 7.5. After removal of a small brownish precipitate by centrifugation (35,000*g*), the supernatant liquid is brought to 0.58 saturation, at pH 7.5. The precipitate is collected by centrifugation (35,000*g*, 60 min) and dissolved in 0.01 M EDTA (Na^+)–0.01 M mercaptoethanol (pH 7.5). The $(NH_4)_2SO_4$ concentration is again estimated, adjusted to 0.30 saturation, and applied to a 1.5 × 90 cm column (Pharmacia) packed with Sephadex G-75 (later studies employed G-100) equilibrated with 0.30 saturation $(NH_4)_2SO_4$ (1.17 M) in 0.01 M EDTA–0.01 M mercaptoethanol (pH 7.5). A flow rate of 0.37 ml/min (maintained with either a hydrostatic head, or with a peristaltic pump) is employed to displace the enzyme peak, monitored at 280 mμ. A very small minor peak may appear near the excluded volume of Sephadex G-100 followed closely by the major peak containing the enzyme, and finally a trace diffuse peak. Tubes is containing the major portion of the enzymatic activity (major component) are combined and the protein is concentrated by increasing the $(NH_4)_2SO_4$ concentration from 0.30 to 0.90 saturation (pH 7.4). The precipitated enzyme can stand for 4–6 hr without loss of activity after which it is centrifuged (35,000*g*, 56 min) and the pellet is dissolved in a minimum volume (1.5–3.0 ml) of 0.01 M EDTA–0.01 mercaptoethanol (pH 7.4), such that the final protein concentration will lie between 75 and 90 mg per ml (fraction VI).

Crystallization. Crystallization is conducted in a dialysis bag and is induced by a slow adjustment of $(NH_4)_2SO_4$ concentration *via* dialysis, in a closed container filled with liquid (with the gas phase eliminated) and with the dialysis fluid saturated with N_2. The protein solution is dialyzed with magnetic stirring *vs.* a solution of 0.40 saturation $(NH_4)_2SO_4$ in 0.01 M EDTA–0.01 M mercaptoethanol (pH 7.4). (The $(NH_4)_2SO_4$–EDTA solution is saturated with N_2 gas which has been washed with water, before the final addition of β-mercaptoethanol.) After 24 hr, the $(NH_4)_2SO_4$ concentration is increased by 0.01 saturation, and if a small amorphous precipitate appears, it is removed by centrifugation, otherwise the dialysis is continued for 12 hr and finally, the saturation is increased to 0.42. At this point, the viscosity of the solution usually shows a noticeable increase followed by incipient crystallization within 12–26 hr. The enzyme crystallizes in the form of relatively large octahedrons (Figure 2) which tend to settle in the dialysis bag. After standing undisturbed (without stirring), qualitatively one observes that the maximum yield of enzyme in crystalline form is usually obtained after a large crop of the crystals had settled and the mother liquor above the settled crystals appears clear but with now a noticeable decrease in viscosity. (Sometimes this occurs by increasing the $(NH_4)_2SO_4$ saturation by an additional 0.005–0.02.) Recrystallization is conducted in the same fashion and is continued (two to three times) until the specific activity of the mother liquor approaches that of the crystalline enzyme.

Results and Discussion

A summary of the data obtained on a typical preparation (preparation 10) on a 6-kg scale is given in Table I. The procedure permits the isolation of the enzyme after some 200-fold purification, and in reasonable yield. It often leads to a final preparation which has approximately a specific activity of *ca.* 130–140 units/mg, in terms of the apparent second-order velocity constant employed earlier for the crystalline rabbit muscle enzyme (Kuby *et al.*, 1954) or *ca.* 240–250 units/mg at 30°, when assayed by the pH-Stat procedure, also developed originally for rabbit muscle enzyme.

It is apparent (Table I) that during the initial steps of the preparation, the enzyme is activated by reducing agents (*e.g.*, cysteine, dithiothreitol, dithioerythritol, or β-mercaptoethanol has been similarly employed) and which is in agreement with the observations of

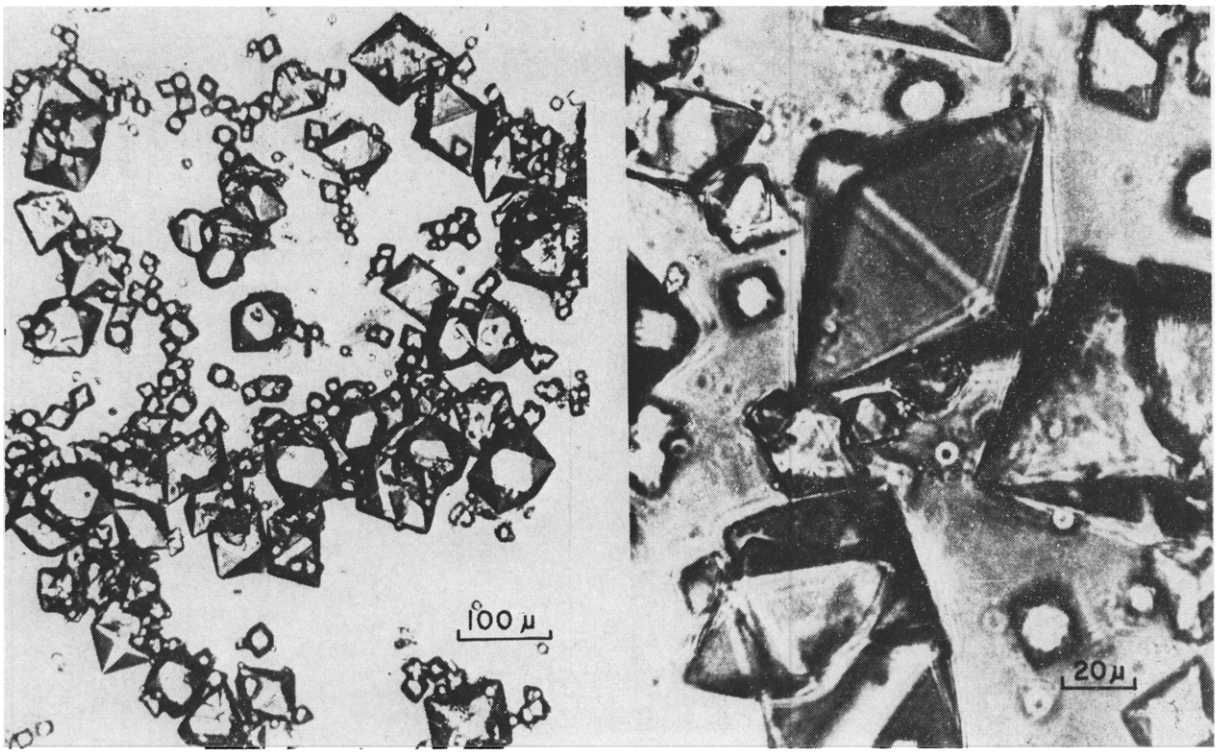

FIGURE 2: Crystals of calf brain ATP–creatine transphosphorylase. The photomicrographs were obtained with a Zeiss phase-contrast microscope at a temperature of 3°. The magnifications are indicated by the 100- and 20-μ lines drawn on the respective photographs.

Wood (1963a) on the ox brain enzyme. At the point where β-mercaptoethanol had been introduced (fraction V), and the enzyme presumably "reduced," the activation disappears and in fact a slight inhibition is sometimes noticeable with cysteine as a diluting reagent, possibly the result of mixed disulfide formation since both thiols and their oxidation products would be present; thereafter, β-mercaptoethanol is retained for dilution of the enzyme prior to assay.

The conditions used for extraction and the low-temperature fractionation step in the presence of Mg^{2+} are based on earlier observations (Kuby and Mahowald, 1958); the direct addition of aqueous ethanol at low temperatures to the homogenate facilitates further extraction of the enzyme, and enzyme assays on the high-speed supernatant liquid of the homogenate (fraction I) are likely to be low. The initial extraction, ethanol steps, and dialysis solve many of the problems (but not all) concerned with phospholipid removal and permit further purification of the enzyme. The use of phosphocellulose has a twofold purpose. It separates the bulk of the contaminating hemoglobin-like proteins as well as neutral and basic proteins from the enzyme (at an early stage); and would completely separate any possible traces, should they be present, of muscle-type ATP–creatine transphosphorylase from brain type (as will be made clearer in a later communication on the calf muscle enzyme), as a result of the enormous differences in charge distribution between the two enzymes (Yue *et al.*, 1968). To avoid the possibility of the brain enzyme, separating into several fractions (*e.g.*, by virtue of different stages of oxidation), mercaptoethanol is introduced no earlier than at the point of the DEAE-cellulose chromatography. Prior to this point, the purification procedure is designed to isolate in good yield the total enzymatic activity, and should there be more than one brain isoenzyme, this separation would be effected by the terminal stages of purification *via* chromatography. Tris–succinate buffers, which Peterson and Chiazze (1962) had successfully employed for separating serum protein by gradient chromatography on DEAE-cellulose, provide an excellent resolving system for the present case, which is based primarily on ionic strength (*i.e.*, gradients in succinate ion) under controlled pH conditions where the enzyme is maximally stable. The relatively low isoelectric point of the enzyme (Yue *et al.*, 1968) makes the coupled use of phosphocellulose, followed by DEAE-chromatography, at suitable pH values, the method of choice. Sephadex gel filtration is primarily employed to remove traces of contaminating material and trace products which possibly are derived from the enzyme (*via* oxidation, denaturation, or other conformational changes) and which make the crystallization step difficult. The final removal of small protein contaminants is then achieved *via* crystallization (Figure 2) which proceeds easily and reproducibly at this stage of purification, provided the precautions are taken to stabilize the enzyme as outlined above. The relatively large dimensions of the crystals shown, and the fact that they do not appear to have greatly dissimilar dimensions along their three geometric axes, may make them amenable to crystallo-

TABLE I: Fractionation of Calf Brain ATP–Creatine Transphosphorylase (Preparation 10) Initially at 6.4 kg of Tissue (Wet).

Fraction No.	Vol (ml)	Protein Concn (mg/ml)	Total Act. (units × 10^{-4})	Sp Act. (units/mg of protein)	Sp Act., pH-Stat Method[e] (units/mg)	Purification		Recov of Act. (%)	
						Over-All	Over Preceding Step	Over-All	Over Preceding Step
I. Homogenate in 0.1 M ammonium acetate (pH 9.5)	13,209[a]	8.9	7.7[b] 15.2[c]	0.65[b] 1.3[c]	1.91			100[b] 100[c]	100[b] 100[c]
II. 50–70% (95%) ethanol precipitate, −10°, 0.015 M $MgSO_4$	2,211	5.98	8.9 12.1	6.75 10.3	13.1	10.4 5.3	10.4 5.3	116 80	116 80
III. 43–67% saturation $(NH_4)_2SO_4$ precipitate	66.4	35.1	4.2 8.2	18.3 35.5	40.1	28.2 26.9	2.7 3.4	54.5 54	47.5 67.6
IV. Phosphocellulose eluate	38.5	34.45	4.9 7.2	37.6 54.5	89.1	57 42	2.0 1.5	63.7 47.5	116 87.5
V. DEAE-cellulose chromatography (0.001 M mercaptoethanol) followed by concentration	378	2.08	6.9 6.7	88 85	160	135.5 65.5	2.4 1.56	89.5 44	96 93
VI. 40–58% saturation $(NH_4)_2SO_4$ followed by Sephadex G-75 (0.01 M mercaptoethanol)	5.5	89	5.8 5.3	117 108	229	180 83	1.33 1.27	75.3 34.8	84 78
Crystallization									
1. Crystals	2.9	117.3	4.1[d]	118[d]	211	182[d]	1.01	53.3[d]	77.5
2. Crystals	2.0	106.5	2.9	136.1		209.4	1.15	37.7	70.7
3. Crystals	1.6	85.5	1.9	138.9	243[f]	213.7	1.02	24.7	65.5
4. Mother liquor	1.62	16.47	0.27	103					

[a] Supernatant liquid volume calculated from the recovery of an aliquot of homogenate subjected to high-speed centrifugation. [b] Upper numbers refer to results obtained from aliquots of respective fractions (see text, 1 unit = k' = 1 ml/μmole per min) diluted initially in 0.001 M glycine (pH 9.0) at 5° and corrected for slow ATPase activity; following fraction II, the ATPase could be neglected. [c] Lower numbers refer to cases where aliquots of respective fractions were diluted initially in 0.001 M cysteine–0.001 M EDTA (pH 7.0). [d] Enzymatic assays conducted on aliquots diluted initially in 0.1 M mercaptoethanol–0.001 M EDTA (pH 7.4); purification and recoveries are calculated from upper sets (*b*) in third and fourth columns. Thereafter, all assays for crystals are conducted in a similar fashion. [e] 1 unit = 1 μequiv/min. [f] Second and third crystals not assayed by pH-Stat for preparation 10; for preparation 8, third crystals when assayed by procedure described, had a specific activity of *ca.* 243 μequiv min^{-1} mg^{-1}.

graphic analyses; especially, since with care at the third crystallization stage, much larger crystals than those shown in Figure 2 may be readily grown.

Stability Properties. Not all the difficulties have been completely overcome, nor understood as yet, in regard to stabilizing the enzyme in solution so as to conveniently permit a systematic physical–chemical and kinetic characterization of the native molecule. However, a few stability properties have been outlined, at least in regard to the effect of pH and thermal stability. Thus, in Figure 3 are given the results obtained in terms of relative activity, by exposing the enzyme (at 0.45 mg/ml) for 30 min, to various pH values at two temperatures (0 and 35°) and in the presence and absence of β-mercaptoethanol. The enzyme appears to be relatively acid labile, and below pH values of 5.5–6, it appears to rapidly denature at 35°, with concomitant aggregation and precipitation, or similarly at 0°, below pH values of 5–5.5. The addition of 0.01 M mercaptoethanol enhances its thermal stability and its addition would permit physicochemical measurements on the native molecule, even at 35° within the pH range of 6–8 (above or below this range, precipitation is evident), or approximately 5.5–10 at 0°. At an intermediate temperature (25°) and not shown in Figure 3, the results lie intermediate to those shown, and denaturation, as revealed by precipitation and loss in activity, is evident above pH 9 and below *ca.* pH 6. Qualitatively, the enzyme molecule may apparently be attacked by strong oxidants and possibly converted into various conformational states, aggregates, or dissociated products as evidenced by the multibanded results which have been obtained with discontinuous gel electrophoresis studies conducted on persulfate-polymerized polyacrylamide gels *vs.* single-banded patterns with riboflavin photochemically polymerized gels (Yue *et al.*, 1968); however, more quantitative data on the effect of strong oxidants (*e.g.*, persulfate) are lacking at the present. Some interesting observations, with respect to the effect of molecular O_2 on the reactive sulfhydryl groups, will be described later. The rabbit muscle enzyme displays an unusual stability even at elevated temperatures to aqueous ethanol at low ionic strengths and slightly alkaline conditions (Kuby *et al.*, 1954). But this property is *not* shared by the calf brain enzyme and care must be exercised in the ethanol fractionation step (see above) not to exceed the temperature limits denoted. However, at −10°, the enzyme will withstand prolonged treatment with 70% ethanol.

Provided certain instability problems are recognized, it has been shown (Yue *et al.*, 1968) that the crystalline enzyme satisfied a number of physical criteria of purity. For kinetic studies it has been found convenient to store frozen samples of enzyme solutions in liquid nitrogen where losses in enzymatic activity are minimal over prolonged periods of time.

A future comparison of the calf brain enzyme with the muscle ATP–creatine transphosphorylases (which have now been isolated in crystalline form in this laboratory from rabbit man, and calf; Jacobs *et al.*, 1968) should prove to be of interest and of an aid in a final correlation of structure and enzymatic function, and in developing, if possible, unified concepts in regard to their mechanism(s) of action. Further, it will undoubtedly be of interest to compare physically, chemically, and kinetically, the brain ATP–creatine transphosphorylase with its muscle enzyme counterpart from the same species; and to date, in addition to the brain and muscle enzyme from calf, the respective enzymes from rabbit and human brain have also been obtained in crystalline form in this laboratory, to facilitate such studies. One may hope that from such studies, information may eventually be derived which will permit extrapolations to and estimations of the chemical and kinetic behavior of these enzymes within their natural physiological environment. For this hope, and a systematic study of the human isoenzymes, perhaps a more clear understanding of the human genetic disorder of Duchenne muscular dystrophy (Tyler, 1966) may also be forthcoming.

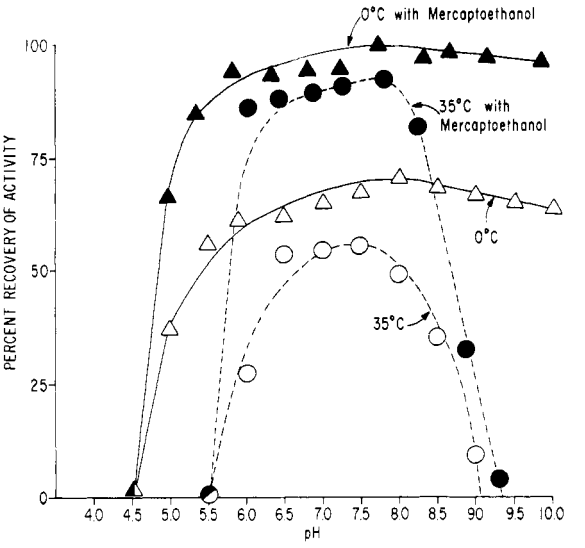

FIGURE 3: The effect of pH on the stability of the ATP–creatine transphosphorylase from calf brain, at two temperatures (0 and 35°), with and without the presence of 2-mercaptoethanol. (▲——▲) 0° for 30 min with 0.01 M 2-mercaptoethanol; (△——△) 0° for 30 min without mercaptoethanol; (●——●) 35° for 30 min with 0.01 M mercaptoethanol; (○——○) 35° for 30 min without mercaptoethanol. Buffers employed: pH 10, 9.5 and 9.0–0.1 M glycine (NaOH); pH 8.5, 8.0, 7.5, and 7.0–0.1 M Tris (HCl); pH 5.0, 5.5, 6.0, and 6.5–0.1 M succinic acid (NaOH); pH 4.5, 4.0–0.1 M acetic acid (NaOH).

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