Brain Isoenzyme of Creatine Kinase

I. PURIFICATION OF RABBIT ENZYME AND PRODUCTION OF SPECIFIC ANTIBODIES*

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Creatine kinase (EC 2.7.3.2) of rabbit brain (BB) was purified to homogeneity as judged by sedimentation velocity analysis in the ultracentrifuge. Nevertheless, the purified enzyme contained as many as seven extraneous protein species when examined by disc electrophoresis in polyacrylamide gel. The purified preparation also showed antigenic heterogeneity when it reacted in agar gel double immunodiffusion analysis with antibodies produced against it in the rooster. At least some of the extraneous proteins appeared to arise from BB during storage. Subsequently BB was purified to homogeneity by disc electrophoresis in polyacrylamide gel. It was excised from the gel and injected repeatedly with Freund's complete adjuvant into roosters. A single immunoprecipitin line was noted in agar gel double immunodiffusion analysis when partially pure enzyme reacted with the antibodies raised to the electrophoretically pure protein. These antibodies inhibited the enzymatic activity of BB in both soluble and insoluble immune complexes. They did not, however, form immunoprecipitates with or inhibit rabbit muscle (MM) or rabbit hybrid (MB) creatine kinases. The antibodies were therefore specific for BB and did not react with MM or MB.

Several methods for purification of creatine kinase and of mammalian brain have been reported (1–5). Two of these reports have described isolated procedures for the rabbit brain enzyme (2, 5). We now report a modification of the method. However, due to the high levels of creatine kinase activity encountered during purification, it was routinely necessary to dilute the enzyme fractions prior to assay. The diluent used was ice-cold 0.01 M Trisacetate, pH 7.2,

EXPERIMENTAL PROCEDURES

Materials

Fresh frozen rabbit brains were obtained in 0.5-kg packages (approximately 60 brains) from Pel-Freez Biologicals, Inc. (Rogers, Ark.). The brains were from rabbits of mixed sex and breed and were not specifically stripped free of dura or blood vessels. Upon arrival at the laboratory, the frozen brain was stored at −20° until used. Rabbit skeletal muscle creatine kinase, containing the isoenzymes MM and MB, was purchased from Sigma Chemical Co. (St. Louis, Mo.).

All reagents, buffers, and dialysis fluids were prepared with doubly distilled deionized water. Ethanol fractionations were performed with absolute ethanol and are expressed as volume per cent assuming that the volumes are additive. Volumes of ethanol were measured at 25°. 2-Mercaptoethanol was obtained from Eastman Kodak Co. (Rochester, N. Y.). It was calculated from the density of 2-mercaptoethanol.

The following reagents for disc electrophoresis in polyacrylamide gels were obtained from Bio-Rad Laboratories (Richmond, Calif.): acrylamide, N,N'-methylenebisacrylamide, N,N',N',N'-tetramethylethylenediamine (TEMED), ammonium persulfate, riboflavin and 5-amin-

naphthalene sulfonate (Mg2+) were from Eastman Kodak Co. (Rochester, N. Y.). Amido black was a product of Hartman-Leddon Co. (Philadelphia, Pa.). The reagents for the histochemical stain used to detect creatine kinase activity following electrophoresis were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Reagents for the creatine kinase assay have been described previously (9). Crystalline bovine serum albumin was used as a standard for protein determination and was a product of Sigma Chemical Co. (St. Louis, Mo.). All other reagents were of analytical grade.

DEAE-cellulose (Cellex D, Bio-Rad Laboratories, Richmond, Calif., approximately 0.67 meq/g) which had been prepared by the method of Peterson and Sober (10) was stored in sealed containers at 2° as a moist filter cake. In this condition, the ion exchanger remained bacteriologically sterile and chemically active for more than a year.

Sephadex G-100 (Pharmacia Fine Chemicals, Toronto, Ontario) was swollen and prepared for use according to the manufacturer's instructions.

Freund's complete adjuvant was purchased from Difco Laboratories (Detroit, Mich.) and agar (Ionagar) was a product of Oxoid Ltd. (London, England).

Creatine Kinase Assay

Creatine kinase activity was assayed (9) at the various stages of purification without modification of the method. However, due to the high levels of creatine kinase activity encountered during purification, it was routinely necessary to dilute the enzyme fractions prior to assay. The diluent used was ice-cold 0.01 M Trisacetate, pH 7.2,
containing 0.014 M 2-mercaptoethanol. Endogenous creatine in the fractions was undetectable in the diluted samples.

**Measurement of Protein**

Protein was measured in the initial purification steps by an automated version (11) of the Lowry assay (12). In both instances of purification and in chromatography column eluents, protein was measured according to its absorbance at 280 nm, assuming that a solution of 1 mg of protein/ml has an absorbance of 1.0.

**Purification Procedure**

Unless stated to the contrary, all fractions were maintained at 0.014 M 2-mercaptoethanol and 2°C throughout the purification. Measurements of pH were made at 25°C and maximum centrifugal forces are quoted.

Fraction 1—Frozen rabbit brain (approximately 450 g) was thawed for 10 h and ground in a meat grinder. The minced brain was dispersed in 1600 ml of 0.01 M Tris/HCl (pH 8.0) containing 0.001 M EDTA, and was stirred overnight. Undissolved precipitate was resuspended in 160 ml of 0.05 M Tris/HCl (pH 8.0). The resulting suspension was stirred at -10°C for 30 min, and the supernatant was recovered as described for Fraction 4.

Fraction 2—Solid NH₄Cl was added to the supernatant at -10°C to a final concentration of 0.1 M. The pH was adjusted to 8.0 at 2°C with 5 M NH₄OH. With the homogenate initially at 0°C, ethanol (20%) was added at approximately 300 ml/h to a concentration of 55%. The temperature of the homogenate was allowed to fall slowly to -10°C as the ethanol addition proceeded. At no time did it rise above 2°C. The ethanolic suspension was stirred at -10°C for 30 min, and the supernatant was recovered by centrifugation after decanting centrifugation at -10°C and 2800 x g for 40 min.

Fraction 3—Enough ethanol (20%) was added to the supernatant at -10°C to increase the ethanol concentration to 60%. The 2% MgSO₄ (pH 8.5) was added dropwise with stirring to a final concentration of 0.01 M. The resulting suspension was stirred at -10°C for 30 min. After its recovery by centrifugation as for Fraction 2, the white precipitate was resuspended in 160 ml of 0.05 M Tris/HCl (pH 8.0) containing 0.001 M EDTA, and was stirred overnight. Undissolved material was removed by centrifugation at 70,000 x g for 1 h. The supernatant was dialyzed against 15 liters of 0.01 M Tris/HCl (pH 8.0) for 18 h.

Fraction 4—Solid (NH₄)₂SO₄ was added slowly with stirring to the supernatant to a final saturation of 70%. The pH was maintained above 7.0 with small amounts of 5 M NH₄OH added as required during the (NH₄)₂SO₄ addition. After 24 h the precipitate was collected by centrifugation at 48,000 x g for 40 min. It was dissolved in 90 ml of 0.1 M Tris/HCl (pH 8.0).

Fraction 5—Fraction 4 was loaded onto a column (2.5 x 100 cm) of Sephadex G-100 which had previously been equilibrated with 0.1 M Tris/HCl (pH 8.0). The protein chromatogram was developed by an ascending elution with this buffer at 10 ml/h and fractions were collected. Fractons containing creatine kinase activity were pooled.

The purified enzyme was stored as a precipitate in 70% (NH₄)₂SO₄.

**Analytical Ultracentrifugation**

About 6 mg of precipitated, purified enzyme (Fraction 7) were recovered as described for Fraction 4 and were dissolved at 2°C in 1 ml of 0.05 M Tris/HCl (pH 7.5) containing 0.1 M KC1 and 0.014 M 2-mercaptoethanol. The enzyme solution was dialyzed overnight at 2°C against a a large volume of buffer. The sedimentation pattern and properties of the purified enzyme were examined in a Spinco model E ultracentrifuge (double sector cell) at 16°C and a protein concentration of 4.7 mg/ml. Pictures were taken every 16 min after attaining 59,780 rpm. The sedimentation coefficient was calculated according to the method of Pedersen (13).

**Disc Electrophoresis in Polyacrylamide Gels**

This technique was used as both an analytical and a preparative method for the isolation of creatine kinase. The separating (small pore) gel contained 7.5% acrylamide and 0.2% bisacrylamide. With the following minor modifications, the method was the same as the original procedure of Davis (14): (a) no sample gel was used; (b) the final concentration of TEMED in the polymerization mixture for the separating gel was 0.057%. No TEMED was used in the polymerization mixture for the stacking (large pore) gel; (c) the final concentration of ammonium persulfate in the polymerization mixture for the separating gel was 0.035%.

**Preparative Electrophoresis—About 8 mg of the (NH₄)₂SO₄-precipitated enzyme (Fraction 7) were collected by centrifugation as described for Fraction 4 and dissolved at 2°C in 1 ml of 0.05 M Tris/HCl (pH 8.3) containing 0.014 M 2-mercaptoethanol. The enzyme solution was dialyzed against 50 ml of this buffer with frequent changes for 48 h at 2°C. An equal volume of 40% sucrose in water containing 0.014 M 2-mercaptoethanol (2%) was added to the retentate. This material was subjected to disc electrophoresis on polyacrylamide gels cast as described previously (15). To the surface of each stacking gel at 2°C and 0.1 ml (approximately 0.25 mg) of the dialyzed enzyme solution containing 20% sucrose (see above) was applied carefully from a polyethylene catheter attached to a 20-gauge needle on a 1-mL syringe. Generally about two gels were used for each experiment. Electrophoresis was conducted at 2°C with a constant current of 1.5 mA/gel for 90 min.

After electrophoresis was completed, the gels were removed from the glass tubes and handled with plastic gloves. Two gels were stained for protein with 1% Amido black and 2% 2-mercaptoethanol. The electrophoretically pure enzyme was stored at 2°C for not more than 24 h prior to its use in immunization.

**Analytical Electrophoresis—About 79% of the protein applied to analytical gels varied between 0.01 and 0.25 mg. After electrophoresis, gels were stained for creatine kinase activity or for protein. Gels were also cut longitudinally and half was stained for protein and half for creatine kinase activity. Smaller protein loads (0.01 to 0.05 mg) were applied to gels which were to be stained for creatine kinase activity. A histochemical stain was used to demonstrate creatine kinase activity. This consisted of 10 ml of buffer (7.94 g of glycylglycine, 0.86 g of magnesium acetate 4 H₂O, and 0.36 g of dextrose in water to a final volume of 100 ml, pH 6.0), 0.4 mg of phenazine methosulfate, 4 mg of nitroblue tetrazolium, 8 mg of nitrinitramine adenosine dinucleotide phosphate, 16 mg of ADP, 40 mg of phosphorylcreatine PC, 100 units of hexokinase, and 50 units of glucose-6-phosphate dehydrogenase. A control stain was compounded in like fashion except that phosphorylcreatine was omitted. Staining of gels was well developed after 10 to 15 min at 37°C.

Analytical disc electrophoresis was also performed with separating gels polymerized with riboflavin at a concentration of 0.002% in the polymerization mixture. Other runs were conducted in ammonium persulfate-polymerized gels with enzyme samples containing 0.001 nmol of thiglycolic acid.

**Polyacrylamide Gel Electrophoresis in Presence of Sodium Dodecyl Sulfate**

About 2 mg of the (NH₄)₂SO₄-precipitated enzyme (Fraction 7) were collected by centrifugation as described for Fraction 4 and were dissolved in 2 ml of 0.01 M sodium phosphate buffer (pH 7.0) containing 1% SDS and 1% 2-mercaptoethanol. The enzyme solution was

1 The abbreviation used is: TEMED, N,N,N',N'-tetramethylethylenediamine; SDS, sodium dodecyl sulfate.
albumin, ovalbumin, and soybean trypsin inhibitor. Electrophoresis was then performed according to the method of Weber and Osborn (17) using the following proteins as standards: α1 and α2, subunits of rat tail collagen, bovine serum albumin, ovalbumin, and soybean trypsin inhibitor.

**Immunization of Roosters**

The purified enzyme (Fraction 7) was subjected to disc electrophoresis not more than 24 h before each immunizing injection. In a glass tube the polyacrylamide gel slices were homogenized gently at 2°C with a motor-driven Teflon pestle in 2 ml of 0.1 M sodium phosphate buffer (pH 6.8) containing 0.014 M 2-mercaptoethanol. The homogenate, containing a total of 1 to 2 mg of enzyme, was mixed well with an equal volume of Freund's complete adjuvant. At 10-day intervals this material was injected subcutaneously into the thighs of young roosters. A total of three injections was administered initially followed by booster injections 6 weeks and 6 months later.

Immunization of another rooster was carried out using the purified enzyme of Fraction 7. The enzyme solution was mixed well with an equal volume of Freund's complete adjuvant and subcutaneous injections were made at 10-day intervals (total of three injections).

One week after the initial three injections and 1 week after each booster injection, the birds were bled from the deep alar vein, using vacuum tubes and 20-gauge needles (Vacutainers, Becton, Dickinson and Co., Toronto, Ontario). Initially, clotted and unclotted blood was obtained. Unclotted blood was collected in tubes containing 0.7 ml of 2% disodium EDTA in 0.15 M NaCl. The plasma was recovered by centrifugation and dialyzed overnight against 0.15 M NaCl. Plasma and serum were frozen in 1-ml aliquots and stored at -20°C.

**Characterization of Antibody Response**

The presence and specificity of anti-rabbit BB antibodies in the immune serum or plasma were examined using the techniques of agar gel double immunodiffusion and antibody inhibition of enzyme activity.

**Agar Gel Double Immunodiffusion**—Double immunodiffusion analysis was performed in 1% agar which was prepared in the following buffer at 96-100°C: 0.01 M Na2HPO4, pH 7.4 (adjusted with 1 N HCl), containing 0.1% sodium azide and 2% NaCl (18). Four milliliters of the hot buffered agar were pipetted into level Petri dishes (5 cm diameter) which had been precoated with 0.1% agar in 0.15 M NaCl. The plasma was recovered by centrifugation and dialyzed overnight against 0.15 M NaCl. Plasma and serum were frozen in 1-ml aliquots and stored at -20°C.

The purified enzyme (Fraction 7) was subjected to disc electrophoresis not more than 24 h before each immunizing injection. In a glass tube the polyacrylamide gel slices were homogenized gently at 2°C with a motor-driven Teflon pestle in 2 ml of 0.1 M sodium phosphate buffer (pH 6.8) containing 0.014 M 2-mercaptoethanol. The homogenate, containing a total of 1 to 2 mg of enzyme, was mixed well with an equal volume of Freund's complete adjuvant. At 10-day intervals this material was injected subcutaneously into the thighs of young roosters. A total of three injections was administered initially followed by booster injections 6 weeks and 6 months later.

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**TABLE I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total enzyme activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Homogenate</td>
<td>1890</td>
<td>170</td>
<td>36300</td>
<td>4.7</td>
<td>100</td>
</tr>
<tr>
<td>2. 55% ethanol precipitate</td>
<td>3590</td>
<td>110</td>
<td>1940</td>
<td>57</td>
<td>65</td>
</tr>
<tr>
<td>3. MgSO4, precipitate redisolved and dialyzed</td>
<td>220</td>
<td>102</td>
<td>1920</td>
<td>101</td>
<td>60</td>
</tr>
<tr>
<td>4. (NH4)2SO4, precipitate</td>
<td>20</td>
<td>105</td>
<td>571*</td>
<td>184</td>
<td>62</td>
</tr>
<tr>
<td>5. Pooled active fractions from first Sephadex G-100 column</td>
<td>90</td>
<td>94.9</td>
<td>172*</td>
<td>552</td>
<td>56</td>
</tr>
<tr>
<td>6. Pooled active fractions from DEAE-cellulose column</td>
<td>140</td>
<td>74.1</td>
<td>66*</td>
<td>1124</td>
<td>44</td>
</tr>
<tr>
<td>7. Pooled active fractions from second Sephadex G-100 column</td>
<td>38</td>
<td>66.4</td>
<td>47*</td>
<td>1412</td>
<td>39</td>
</tr>
</tbody>
</table>

* Determined by the automated adaptation (11) of the method according to Lowry et al. (12).

**RESULTS**

**Purification of Enzyme**—A summary of the purification results appears in Table I. From 450 g of rabbit brain, the final yield was 47 mg of protein, representing 39% of the total creatine kinase activity present in the crude homogenate. The specific activity of the final product was 1412 units/mg which was a 300-fold enrichment over the crude homogenate.

Attempts to purify rabbit brain creatine kinase by the procedure of Eppenberger et al. (2) produced only a 20 to 30% yield of enzyme activity in the supernatant of the ethanol fraction. A partial explanation for their low yield was that only 75% of the total enzyme activity in the crude homogenate was extractable with 0.01 M KCl at 2°C (Table I). In addition, warming the 60% ethanol fraction to 20°C for 2 h yielded one-third to two-thirds less supernatant creatine kinase activity than was present at -10°C and there was no rise in specific activity.

The enzyme was eluted from all chromatographic columns as a single peak of activity. When chromatography on Sephadex G-100 was substituted for the second dialysis recommended by Eppenberger et al. (2), further purification was achieved in addition to desalting (Table I). Loading the DEAE-cellulose column with the unconcentrated pool (90 ml) of enzyme fractions from the Sephadex G-100 column produced satisfactory chromatographic resolution (Fig. 1). The peak of enzyme activity was consistently eluted by salt solution having a conductivity of about 15 mohm. A protein peak, lacking creatine kinase activity but having a pink color, was a constant finding prior to salt gradient elution. The final chromatography was then performed according to the method of Weber and Osborn (17) using the following proteins as standards: α1 and α2, subunits of rat tail collagen, bovine serum albumin, ovalbumin, and soybean trypsin inhibitor.
Specific Antibodies to Purified Rabbit Brain Creatine Kinase

ogram on Sephadex G-100 (Fig. 2) showed a single sharp peak of enzyme activity, contaminated on both shoulders. Amounts of these contaminants varied from batch to batch and, on some occasions, they were not apparent.

Heterogeneity and Stability of Purified Enzyme—In the analytical ultracentrifuge, the purified enzyme sedimented as a single peak (Fig. 3) with an observed sedimentation coefficient of 4.907 S. This value compares favorably with the one reported by Dawson et al. (19). However, a maximum of seven minor protein bands could be demonstrated by disc electrophoresis in polyacrylamide gels (Fig. 4). Of these bands, five had lower and two had higher electrophoretic mobilities than did the major component. Not all batches contained the faster moving proteins. Creatine kinase activity was detected in the major component but was also present in the slower moving minor bands.

The number of minor bands seen after polyacrylamide gel disc electrophoresis was considerably less when the running gels were polymerized with riboflavin instead of ammonium persulfate. Nonetheless, multibanded patterns were consistently observed. In the presence of thioglycolic acid, electrophoresis performed on ammonium persulfate-polymerized gels produced a pattern no different from that observed in the absence of the reducing agent.

After the purified enzyme (Fraction 7) was stored for 2 weeks at 2°C in 0.1 M Tris/HCl (pH 8.0) containing 0.014 M 2-mercaptoethanol, there was a dramatic increase in two of the bands having lower mobilities than the major component (Fig. 4, see arrows). There was a corresponding decrease in the major band. No such changes occurred during the first 24 h of storage but they were evident after 3 days. When the purified enzyme was stored as the (NH₄)_2SO₄ precipitate at 2°C for 2 weeks, the increase in these two minor bands was not observed. Even after 8 weeks storage, these two minor bands were only minimally increased.

Table II indicates the preservation of enzyme activity observed when Fraction 7 was stored for 2 weeks under three different conditions: 2°C, -20°C, and 2°C as a precipitate in the presence of (NH₄)_2SO₄ (70% saturation). Again, stability was greatest when the enzyme was stored as the (NH₄)_2SO₄ precipitate.

The minor proteins of lower electrophoretic mobility appeared on the front shoulder of the peak from the second Sephadex G-100 column. Those of higher mobility were present on the back shoulder.

In the presence of 0.1% SDS and 0.1% 2-mercaptoethanol, polyacrylamide gel electrophoresis revealed a major band of protein with a molecular weight of 42,000. Eight minor protein bands, ranging in molecular weight from 95,000 to 21,000 were visualized when larger quantities (0.1 to 0.2 mg) of protein were applied to the gels.

When roosters were immunized with purified rabbit brain

![Fig. 1 (left). Linear salt gradient elution of rabbit brain creatine kinase (CK) at 2°C from column (2.5 x 40 cm) of DEAE-cellulose. Starting buffer was 0.1 M Tris/HCl (pH 8.0) containing 0.014 M 2-mercaptoethanol. Linear salt gradient established by continuously adding 1 liter of 0.5 M NaCl in starting buffer to 1 liter of starting buffer. Flow rate was 50 ml/h (descending elution) and 5-ml fractions were collected. Solid line denotes absorbance at 280 nm and broken line indicates creatine kinase activity. Gradient (x-x) was measured using conductivity meter. Pool of enzyme-active fractions was made from effluent between 1025 and 1175 ml.](http://www.jbc.org/)

![Fig. 2 (right). Chromatography of rabbit brain creatine kinase (CK) at 2°C on column (2.5 x 100 cm) of Sephadex G-100. Buffer was 0.1 M Tris/HCl (pH 8.0) containing 0.014 M 2-mercaptoethanol. Flow rate was 10 ml/h (ascending elution) and 2.5-ml fractions were collected. Solid line denotes absorbance at 280 nm and broken line indicates creatine kinase activity. Fractions containing the enzyme peak (effluent between 192.5 and 230 ml) were pooled.](http://www.jbc.org/)
creatinine kinase (Fraction 7), antibodies to at least three antigens were detectable by agar gel double immunodiffusion analysis.

Antibodies to Electrophoretically Pure Enzyme - Immunization with the electrophoretically pure enzyme was successful in both roosters. Each immune plasma reacted with partially pure rabbit brain creatine kinase (Fraction 5) and yielded a single sharp precipitin line in agar gel double immunodiffusion analysis (Fig. 5). No reaction was observed when the preimmune plasmas of these animals were used instead (Fig. 5). The use of immune serum in lieu of immune plasma did not alter the antigen-antibody reaction in these immunodiffusion studies.

After the more responsive rooster had received a fifth injection of electrophoretically pure rabbit brain creatine kinase, its immune plasma continued to show a single immunoprecipitation reaction with partially pure enzyme. Serial dilution of either the immune plasma or the antigen failed to indicate any other precipitin lines.

Antibody Specificity for Brain Isoenzyme - No immunoprecipitation reaction in agar gel was observed between the anti-BB immune plasma and partially purified MM or MB of rabbit brain creatine kinase (Fraction 5) at concentration of 1 mg/ml; when BB reacted with the anti-BB immune plasma, the precipitin line formed in agar gel could not be stained for enzyme activity.

After the anti-BB immune plasma was incubated with rabbit brain extract for 1 h at 25°C, a small amount of precipitate was formed. The amount of precipitate was markedly increased after an additional incubation of 18 h at 2°C. No precipitate appeared during the same experiments performed with nonimmune rooster plasma.

Fig. 6 illustrates the effects of anti-BB immune plasma on the supernatant creatine kinase activity of rabbit brain and skeletal muscle extracts after the combined incubation period. The pattern of attenuation in BB activity was fully manifested after the initial incubation period (1 h at 25°C) and without removal of precipitate by centrifugation. Anti-BB immune plasma did not inhibit skeletal muscle creatine kinase activity (MM and MB) nor partially purified MB of rabbit heart.

**DISCUSSION**

**Purification Procedure** - The purification of rabbit brain creatine kinase is a more difficult procedure than the purification of the muscle isoenzymes. BB is more susceptible to denaturation during purification, especially during the ethanol fractionation. Our recovery of activity (40%) is twice that reported by Eppenberger et al. (2) without any apparent sacri-

**Table II**

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Recovery of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 days</td>
</tr>
<tr>
<td>2°C</td>
<td>74</td>
</tr>
<tr>
<td>-20°C</td>
<td>74</td>
</tr>
<tr>
<td>2°C and (NH₄)₂SO₄ (70%)</td>
<td>94</td>
</tr>
</tbody>
</table>

**Fig. 6**. Effect of anti-rabbit BB immune rooster plasma on creatine kinase (CK) activity of rabbit brain (●) and gastrocnemius (○) extracts. Varying dilutions of extracts were added to a fixed amount of immune plasma (constant final volume). Incubation was for 1 h at 35°C and 18 h at 2°C. Residual creatine kinase activity was measured in 2000 x g supernatant. Note lack of inhibition of creatine kinase activity in gastrocnemius extract which contained MM and MB activities but only traces of BB activity. Same result was noted for partially purified MB of rabbit heart. Creatine kinase activity of brain extract, which contains only BB, was inhibited. These results demonstrate specificity of anti-BB immune plasma. Effect on rabbit brain creatine kinase was not due to precipitation since it was fully manifest after 1 h at 25°C when precipitation was minimal.
duced by sample blanks, this was not due to an initial overestimation of creatine kinase activity in the homogenate. By contrast, Wood (1) found virtually all the creatine kinase activity of ox brain to be soluble in 0.01 M KCl containing 0.001 M EDTA. However, Swanson (20) has shown that when guinea pig brain is homogenized in 0.32 M sucrose, 25% of the total creatine kinase activity is particle-bound, probably to mitochondria. Sullivan et al. (4) found that only 60% of rat brain creatine kinase was extractable in 0.25 M sucrose and only 80% was extractable in water. Jacobs and Lehninger (21) reported that acetate or phosphate ions in the presence of Na⁺ but not K⁺ were necessary for the extraction of rat heart mitochondrial creatine kinase. Keutel et al. (5) extracted rabbit brain creatine kinase in 0.1 M ammonium acetate. Possibly their high yield is related to a more completed extraction of the enzyme. Based on the actual creatine kinase activity which was initially extractable, our recovery of activity was 51%.

**Heterogeneity of Purified Enzyme**—After ethanol and salt fractionation and column chromatography, the enzyme preparation contained variably small amounts of contaminants when examined by polyacrylamide gel disc electrophoresis. Yue et al. (22) reported similar results for the calf brain enzyme. However, they noted an absence of multibanded patterns when the gels were polymerized with riboflavin instead of ammonium persulfate. We failed to confirm this finding nor could we eliminate the multibanded patterns in persulfate-polymerized gels by performing the electrophoresis in the presence of thioglycolic acid as advocated by Brewer (23). At least some of the contaminants in our preparation were clearly not electrophoretic artifacts since some could be detected on the front shoulder of the enzyme peak from the final Sephadex column, some increased on storage and some were antigenic.

An extraneous protease might be responsible for the heterogeneity of the purified enzyme (24). Proteolytic activity could certainly account for the smaller molecular weight fragments and association of small fragments with the native enzyme could produce the higher molecular weight contaminants. The data obtained from SDS-polyacrylamide gel electrophoresis are also consistent with this possibility. While the expected amount and size of the native subunit were observed, there was also a number of other polypeptides ranging in molecular weight from 95,000 to 21,000. Some of the smaller molecular weight components may have been related to the smaller molecular weight contaminants on the back shoulder of the Sephadex column. In keeping with the protolytic hypothesis, some of these small molecular weight peptides could be fragments of the native subunit. The larger molecular weight components noted in the SDS-gels might be incompletely dissociated forms of the BB isoenzyme, including the native enzyme, the native enzyme plus fragments or residual, partially degraded forms of the native enzyme.

Lastly, some of the contaminating proteins in our purified preparation may have arisen during the purification procedure as in vitro conformational variants of the native enzyme. Watts (25) has concluded that "the ability to adopt more than one kinetically stable conformation is inherent to some degree in all brain-type creatine kinases."

**Antibody Response and Specificity**—Hartman and Udenfriend have reported the high degree of antibody specificity that results from immunizing with an enzyme excised from polyacrylamide gel after disc electrophoresis (16). Polyacrylamide clearly does not interfere with antibody response and may well act as an adjuvant (26). We have confirmed these observations.

In our experiments the antibody response was specific for the BB isoenzyme as neither MM or MB reacted with anti-BB immune plasma in double immunodiffusion or enzyme inhibition studies. Bulcke and Sherwin (27) showed an immunoprecipitation reaction between rooster anti-rabbit MM antisemur and rabbit MM but not between this antisemur and rabbit BB. They suggested that the anti-MM antisemur inhibited both MM and MB of rabbit heart but, according to their data, the overall inhibition of creatine kinase activity for heart was less complete than for muscle. Furthermore, the thermal stability of rabbit MB is uncertain and a portion of the MB activity could have been destroyed by the conditions of their incubation (37°C for 1 h). Using their antisemur, we could not demonstrate any immunoprecipitation reaction in double immunodiffusion analysis with partially purified MB of rabbit heart.

Eppenberger et al. (2) reported weak immunoprecipitation and complement fixation reactions between chicken MB and rabbit anti-chicken BB or anti-chicken MM. These antisera reacted strongly by both techniques with the homologous antigen but not with the heterologous antigen. No mention is made by these authors as to the purity of the artificially prepared MB isoenzyme. It is possible that their antisera were reacting with contaminating MM and BB isoenzyme proteins, either enzymatically active or inactive. These authors also fail to indicate the relationship of immunoprecipitin lines ostensibly involving MB to those involving MM or BB. Thus, it is not clear from the data they present whether their antisera actually demonstrated immunoprecipitation reactions with MB. Their complement fixation data are more convincing but this immunochromical technique is certainly more sensitive than immunoprecipitation. Maximum complement fixation occurred only at higher antibody and antigen concentrations indicating that MB possessed relatively few antigenic determinants in common with MM or BB.

The absent or decreased reactivity of MB with antibodies to MM or BB is not really surprising. There is no clear evidence to support the idea that antibodies are made to the subunits rather than to the dimer. On the contrary, Bethell et al. (28) have shown by complement fixation that antibodies to aspartate transcarbamylase react with greater activity toward the native enzyme than to either the catalytic or the regulatory subunit.

Immunization with a specific antigen produces a spectrum of antibodies directed against the numerous antigenic sites on the antigen. Some of these antibodies may be detected by precipitation, complement fixation, or changes in antigen function (e.g. enzyme inhibition), etc. The antibodies responsible for each of these phenomena may be quite distinct; their appearance and titers depend on the time course of immunization, the route of immunization, the amount of antigen injected and the host's immune response.

Because we found that BB activity was inhibited in both the soluble and insoluble immune complexes, it is tempting to speculate that the same antibodies are responsible for both inhibition and precipitation. Whether the inhibitory effect is mediated by the same antibodies responsible for precipitation or by distinct (nonprecipitating), inhibitory antibodies cannot be known unless inhibition could be demonstrated in the absence of precipitating antibodies. We have not attempted a fractionation of these antibodies to elucidate this point. It is
possible that inhibition is due to both precipitating and non-precipitating antibodies.

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