Isozymes of Rabbit Phosphofructokinase

ELECTROPHORETIC AND IMMUNOCHEMICAL STUDIES*

MICHAEL Y. TSAI AND ROBERT G. KEMP†

From the Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

SUMMARY

The distribution of phosphofructokinase isozymes in rabbit tissues was analyzed by electrophoretic and immunochemical techniques. Antiserum to the enzyme purified from skeletal muscle and to enzyme purified from liver were prepared. The antiserum to muscle phosphofructokinase showed a partial reaction with the liver enzyme, whereas little cross-reaction was observed with antiserum to liver phosphofructokinase toward the muscle enzyme.

Skeletal muscle extracts and heart extracts contain a single identical isozyme species. It is proposed that this isozyme be designated phosphofructokinase A. The major isozyme species of liver and erythrocyte are also identical (phosphofructokinase B). All other tissues examined contain isozymes that include both A and B monomers. Extracts of lung, adipose tissue, and stomach contain a complete set of five A-B hybrids. Other tissues appear to contain isozymes that have A and B monomers, but both electrophoresis and antiserum precipitation data indicate that at least one other isozyme is present. This is most obvious in extracts of brain tissue. Electrophoresis of extracts of brain produces at least four activity bands of mobility intermediate between those observed following electrophoresis of isozymes A and B. One of these bands of activity was not removed by treatment with a combination of both antisera while a similar treatment of A-B hybrids removed all activity. The residual activity is designated phosphofructokinase C.

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† American Heart Association Established Investigator.
with this buffer. The enzyme eluted as a peak of constant specific activity that was pooled and concentrated by ammonium sulfate precipitation. The enzyme was again crystallized. The specific activity at 26° was 100 i.u. per mg.

Lever phosphofructokinase was prepared from frozen rabbit livers through Step 4 of the published procedure (8). Enzyme used for injection into guinea pigs was purified further. Three preparations were pooled and concentrated by ammonium sulfate precipitation at 0.25 saturation. The precipitated enzyme was dissolved in a minimal volume of 50 mM Tris-phosphate, 2 mM EDTA, 100 mM ammonium sulfate, 0.1 mM ATP, 0.1 mM dithiothreitol (all at pH 8.0), and placed on a column of Bio-Gel A-150 (Bio-Rad Laboratories) previously equilibrated with the same buffer. The major peak of enzyme activity was pooled and concentrated by ammonium sulfate precipitation. The specific activity was 95 i.u. per mg.

Preparation of Hybrids of Phosphofructokinase from Muscle and Lever—Hybrids of the two types of phosphofructokinase were prepared as previously described (9). The procedure involves dissociation of both enzymes at pH 5.3, mixing the two preparations, and raising the pH to 7.0 after 5 min. Approximately 90% of the original activity was recovered.

Preparation of Antisera—For the preparation of antisera to muscle phosphofructokinase the following procedure was employed. Enzyme crystals were collected by centrifugation, dissolved in a minimal amount of 50 mM glycerol-P, 2 mM EDTA (pH 7.2), and dialyzed against this buffer overnight. After dialysis, the enzyme solution was diluted to a protein concentration of 10 mg per ml and was mixed with an equal volume of Freund's complete adjuvant, containing 1% killed M. tuberculosis. A series of adult guinea pigs were injected subcutaneously with 0.15 ml (0.7 mg of enzyme per injection) of this suspension. Similar injections, but with enzyme mixed with incomplete adjuvant (no bacteria), were repeated 2 and 4 weeks later. Two weeks following the last injection, blood was collected by heart puncture, allowed to clot overnight, and centrifuged to obtain the clear serum.

Liver enzyme was similarly mixed with Freund's complete adjuvant and injections of 0.8 mg of enzyme were given to a series of guinea pigs. A second injection with enzyme mixed with incomplete adjuvant was given 3 weeks later. Eleven days following the second injection, blood was collected by cardiac puncture. The blood was allowed to clot overnight and the serum was removed following centrifugation. The respective antisera were tested at various dilutions for their ability to inhibit muscle and liver phosphofructokinases and for the appearance of precipitin lines by the Ouchterlony double-diffusion method (15). The Ouchterlony plates were prepared containing 0.8% agar (Ionautar 2, Colin), 50 mM Tris-phosphate, 2 mM EDTA, 0.1 mM ATP, 0.1 mM ammonium sulfate, 0.1 mM dithiothreitol (pH 8.0). This gel was prepared by heating a 1% solution agar to boiling and then mixing rapidly with 0.25 volumes of a buffer containing the above listed components at five times concentration. The agar was immediately poured into Petri dishes, holes were punched, and the plates were used within 24 hours. Diffusion studies were carried out at room temperature. The rather unusual buffer in the agar was chosen when poor success was achieved in seeking precipitin lines with agar containing saline alone, barbital buffer, or phosphate buffer, probably because of precipitation or aggregation of the enzyme before it penetrated the agar. The resulting buffer was the one routinely used for diluting the enzyme although not all of the components were required to give a good precipitin line. Surprisingly, the presence of ammonium sulfate in the agar was required for detecting precipitin lines with low concentrations of muscle enzyme. The antisera from two guinea pigs that gave indication of having the most potent antibody to muscle phosphofructokinase were combined. In order to remove any small effectors in the serum and to purify partially the globulin fraction, ammonium sulfate was added to a final saturation of 0.5 and the precipitate obtained was dissolved in 50 mM Tris-phosphate, 2 mM EDTA, 0.1 mM ammonium sulfate, 0.1 mM ATP (pH 8.0). Similarly the two most potent antisera to the liver enzyme were purified and dissolved in the same buffer. The volumes of the purified antisera were adjusted to approximately the same volume as the original antisera. Although for all work reported here the partially purified antibody fractions were employed, they will be subsequently referred to simply as antisera.

Control sera were prepared by collecting guinea pig blood, removing the clot, and salting out and dissolving the protein fraction as described for the antisera fractions.

Electrophoresis—Zone electrophoresis was performed at 4° on 12-inch cellulose acetate strips (Shandon) in a buffer system at pH 8.1 that consists of 50 mM glycylglycine, 0.1 mM EDTA, 1 mM ATP, and 0.25 mM dithiothreitol. For most studies, strips that were 50 mm wide were employed, which allowed the placing of markers (purified muscle and liver phosphofructokinase) on the same strip beside the isozyme mixture to be analyzed for a more accurate comparison of electrophoretic mobilities. Following electrophoresis, enzyme activity on the strips was detected by the development of reduced tetrazolium in a thin 0.3% layer of L0-nagard No. 2 containing enzymes and substrates required for coupling of the phosphofructokinase reaction to the generation of DPNH by glyceraldehyde-3-P dehydrogenase (8). The blue bands associated with enzyme activity were visualized and photographed through the inverted glass plate.

Preparation of Tissue Extracts—All tissues were obtained from New Zealand White rabbits killed by an overdose of Nembutal and bled by cutting vessels in the neck. The tissues were rapidly removed, stored in crushed ice, and used for the preparation of extracts immediately. Muscle, heart, liver, kidney cortex, adipose tissue, lung, and stomach from which the mucosa had been stripped were minced with scissors and homogenized in a Waring Blender with 3 volumes of an ice cold buffer consisting of 30 mM potassium fluoride, 4 mM EDTA, and 1 mM dithiothreitol, all at pH 7.5. This buffer will be subsequently referred to an extraction buffer. Two homogenization periods of 45 s each with a 30-s pause between each period were employed. Brain (cerebrum) and spleen were homogenized with 3 volumes of extraction buffer in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Uterus and testes were first minced and then homogenized in a Potter-Elvehjem vessel fitted with a glass pestle with ground glass surfaces. All homogenates were centrifuged at 18,000 x g for 20 min, and the supernatant solutions were passed through glass wool. For the preparation of erythrocyte extracts, blood from rabbits whose neck blood vessels had been cut was collected into a beaker containing a small amount of heparin. The erythrocytes were collected by centrifugation and washed twice with isotonic saline to remove leukocytes and platelets. The cells were suspended in extraction buffer and sonicated for 4 s at 4 amp with a Branson sonifier S 125. The sonicate was centrifuged at 18,000 x g for 20 min and the supernatant removed. Thymic lymphocytes (thymocytes) were obtained from the thymuses of 4- to 5-week-old rabbits. The whole thymus was cut into pieces and suspended in isotonic saline. The tissue was gently disrupted by slowly
squeezing them in a Potter-Elvehjem homogenizer with a loose fitting Teflon pestle. The thymus stroma was allowed to settle and the thymocytes were removed by decanting. The cells were collected by centrifugation at 1000 × g and were resuspended in the extraction buffer. Sonicates of the thymocytes were prepared as described for the preparation of erythrocyte sonicates.

Precipitation of Enzyme with Antisera—Tissue extract or purified enzyme was diluted with extraction buffer to a final enzyme concentration of 0.6 to 1 unit per ml. The diluted enzyme in a volume of 0.1 ml was mixed with 0.2 ml of extraction buffer and 0.1 ml of antiserum that had been diluted to the appropriate concentration with control serum. Control serum was used as a diluent to correct for any activity changes due to the presence of activators or inhibitors in the partially purified serum. No phosphofructokinase was detectable in any of the partially purified sera and no measurable activation or inhibition of muscle or liver phosphofructokinase was observed upon incubation of control sera with the enzymes. The mixture of enzyme and serum was incubated for 5 min at 37° and then centrifuged for 20 min at 15,000 × g.

The enzyme activity in the supernatant solution was assayed at 26° and pH 8.2 in 3 ml of a medium containing 25 mM glycylglycine, 25 mM glycerol-P, 1 mM EDTA, 3 mM (NH₄)₂SO₄, 6 mM MgCl₂, 0.12 mM DPNH, 0.1 mM dithiothreitol, 0.6 unit of aldolase, 0.3 unit of triosephosphate isomerase, 0.3 unit of glycerol-P dehydrogenase, 1 mM ATP, and 1 mM fructose 6-P.

RESULTS

Tissue Distribution of Phosphofructokinase—Table I describes the distribution of phosphofructokinase in extracts of rabbit tissues as measured under optimal conditions. As might be predicted, the activities are highest in those tissues considered to have high rates of glycolysis; i.e. skeletal muscle, heart, and brain. The activities in Table I are those measured in 18,000 × g supernatant fractions of crude homogenates prepared in the presence of 30 mM potassium fluoride, 4 mM EDTA, 5 mM dithiothreitol, all at pH 7.5. The importance of fluoride ion in preparing phosphofructokinase from skeletal muscle has been documented by Ling et al. (16). We have also observed that the activities in extracts of skeletal muscle, heart, and smooth muscle are higher in extracts prepared with fluoride present. Dithiothreitol, however, replaces the requirement for fluoride, and its inclusion with fluoride often leads to even higher activity in crude extracts. Neither fluoride nor dithiothreitol had significant effect on activity recovery in other tissues that were tested with the exception of fetal liver. High concentrations of dithiothreitol were required for optimal recovery of activity in fetal liver extracts, whereas extraction of the enzyme from adult livers did not require dithiothreitol. This is despite the fact that the major part of late fetal liver phosphofructokinase is identical to the adult liver enzyme by several criteria (17).

Precipitation by Antiserum of Purified Phosphofructokinases Isolated from Skeletal Muscle and Liver—Brief incubation of the antiserum against muscle phosphofructokinase with purified muscle enzyme resulted in about 40% inhibition of the activity, whereas very little inhibition of liver phosphofructokinase was observed under these conditions. Similarly, the antiserum against liver phosphofructokinase partially inhibited the liver enzyme. A much clearer demonstration of the formation of the antigen-antibody complex was obtained by centrifugation of antigen-antibody mixtures. Antiserum against phosphofructokinase from rabbit skeletal muscle and liver were effective in the removal of enzyme activity from solutions containing purified enzymes or from crude tissue extracts by centrifugation at 15,000 × g for 20 min. The effect of increasing concentrations of antisera to liver enzyme on the removal of activity from solutions of purified skeletal muscle phosphofructokinase, purified liver enzyme, a mixture of the two isozymes, and a mixture of the hybrids of the isozymes is shown in Fig. 1. The antisera to liver enzyme precipitated about 95% of the phosphofructokinase from liver under conditions where less than 5% of the muscle enzyme was removed from solution. The antisera precipitated more activity from solution of muscle and liver phosphofructokinase hybrids, produced from equal amounts (on an activity basis) of muscle and liver enzyme, than from an unhybridized mixture of the two enzymes. This is reasonable when one considers that a hybridized mixture contains more enzyme molecules containing liver monomers than does a simple mixture of the two enzymes. Antiserum to muscle phosphofructokinase precipitated more of the muscle enzyme than the enzyme from liver, but in this case

### Table I

**Distribution of phosphofructokinase in rabbit tissues**

Extracts were prepared and assays performed as described under "Experimental Procedure." With the exception of the data from the thymus where a 5 week old animal was used, all data were obtained with adult rabbits (5 to 7 lbs).

| Tissue         | Units g wet wt tissue | Units mg extract protein |
|----------------|-----------------------|--------------------------|
| Skeletal muscle| 156                   | 2.7                      |
| Heart          | 22.4                  | 0.35                     |
| Cerebrum       | 10.5                  | 0.26                     |
| Testes         | 5.4                   | 0.093                    |
| Lung           | 4.4                   | 0.070                    |
| Spleen         | 3.1                   | 0.064                    |
| Kidney cortex  | 2.8                   | 0.027                    |
| Liver          | 2.4                   | 0.023                    |
| Stomach        | 2.0                   | 0.038                    |
| Uterus         | 1.4                   | 0.039                    |
| Adipose        | 0.51                  | 0.061                    |
| Erythrocyte    |                       | 0.0641                   |
| Thymocyte      |                       | 0.023                    |

![Fig. 1. Precipitation of purified phosphofructokinases by antiserum against liver phosphofructokinase. Precipitations were carried out as described under “Experimental Procedures” with the indicated amounts of antiserum against liver phosphofructokinase. The enzyme activity remaining in the supernatant fraction was measured. ○, muscle phosphofructokinase; △, liver phosphofructokinase; ▲, 1:1 mixture of liver and muscle enzyme; □, hybridized 1:1 mixture; □, hybridized 2:1 (liver-muscle) mixture. Hybridization procedure described under “Experimental Procedures.”](http://www.jbc.org/doi/10.1074/jbc.251.4.787)
The activity can be accounted for by the enzymes that are typical of muscle and liver. Antiserum to muscle enzyme precipitates muscle enzyme present in hybridized mixtures. The cross-reaction was more pronounced than with antiserum to liver enzyme. Whereas 98% of the muscle enzyme was removed by 50 μl of muscle antiserum, 52% of the liver enzyme was removed by that amount. The amount of liver enzyme removed by the muscle antiserum gradually increased as the amount of antiserum was increased, but even with 200 μl of antiserum, only 50% of the liver phosphofructokinase was precipitated. That the anti-muscle phosphofructokinase serum recognizes some components of liver enzyme was also shown by the Ouchterlony diffusion plates. A weak reaction of partial identity (spur formation) was observed. A summary of the precipitation data with the two antisera is shown in Table II. As with antiserum to liver enzyme, the anti-muscle phosphofructokinase serum precipitates muscle enzyme present in hybridized mixtures.

Characterization of Phosphofructokinase Activity of Various Tissues with Antisera—Various quantities of both antisera were tested for their ability to precipitate phosphofructokinase activity from crude extracts of rabbit tissues. The antiserum to purified liver phosphofructokinase precipitated most of the activity found in liver extracts. Previous electrophoretic analysis of liver extracts indicated that almost all of the phosphofructokinase activity of liver extracts migrated as a single band characteristic of the purified liver enzyme used in the present studies (8). The antiserum was also capable of precipitating almost all of the activity from erythrocyte hemolysates, suggesting that it did not react with the enzyme from liver. This is consistent with the recent study of rabbit erythrocyte phosphofructokinase by Tarui et al. (18). The properties that they describe for the erythrocyte enzyme are strikingly similar to those previously ascribed to the rabbit liver enzyme in a report from our laboratory (8). Practically no precipitation by antisera to liver enzyme of phosphofructokinase was observed. The anti-muscle phosphofructokinase serum precipitated most of the activity in heart and skeletal muscle extracts, supporting a conclusion that the heart and skeletal muscle enzymes are identical.

Electrophoretic Analysis of Tissue Crude Extracts—Previous work from this laboratory has shown that phosphofructokinase from muscle and liver can be readily separated by electrophoresis on cellulose acetate (8). Furthermore, the three hybrids generated following dissociation and recombination of the muscle and liver enzyme can also be separated from the parent isoenzyme (9). The electrophoresis technique was applied to the analysis of phosphofructokinase in crude extracts of rabbit tissues in an attempt to verify the conclusions derived from the antiserum precipitation data. Fig. 2 shows the activity pattern detected following electrophoresis of crude extracts of rabbit skeletal muscle, heart, liver and erythrocytes. All of the activity in heart extracts migrated the same distance as that of skeletal muscle extracts. In like manner, most of the activity of erythrocyte extracts behaved on electrophoresis similar to the activity of liver extracts. This, of course, substantiates the conclusions of the cross-reaction was more pronounced than with antiserum to liver enzyme.

### Table II

| Tissue            | Activity removed by Liver PFK antiserum (%) | Activity removed by Muscle PFK antiserum (%) |
|-------------------|--------------------------------------------|--------------------------------------------|
| Liver PFK         | 96                                         | 32                                         |
| Muscle PFK        | 3                                          | 98                                         |
| Muscle PFK and liver PFK| 46                                      | 69                                         |
| Hybridized mixture| 68                                         | 82                                         |

* Mixture of an equal number of enzyme units of each enzyme.

### Table III

Crude extracts prepared and antiserum precipitations carried out as described under "Experimental Procedure." Results indicate activity removed by the addition of 25 μl of liver phosphofructokinase antiserum or 50 μl of muscle enzyme antiserum.

| Tissue                | Activity removed by Liver PFK antiserum (%) | Activity removed by Muscle PFK antiserum (%) |
|-----------------------|---------------------------------------------|---------------------------------------------|
| Heart                 | 2                                           | 92                                         |
| Skeletal muscle       | 4                                           | 92                                         |
| Liver                 | 32                                          | 25                                         |
| Erythrocytes          | 96                                          | 32                                         |
| Lung                  | 58                                          | 78                                         |
| Stomach               | 58                                          | 67                                         |
| Adipose               | 64                                          | 58                                         |
| Cerebrum              | 11                                          | 50                                         |
| Thymus                | 27                                          | 91                                         |
| Testes                | 43                                          | 52                                         |
| Spleen                | 58                                          | 40                                         |
| Uterus                | 62                                          | 30                                         |
| Kidney cortex         | 72                                          | 32                                         |
likely that at least one additional isozyme is present in these present. It is suggested by the antisera precipitation data, it is often obtained, and up to six bands could be distinguished in liver and muscle bands. Partial resolution of several bands was indicated the presence of muscle and liver isoenzymes and a smear of poorly resolved activity bands that was located between the liver and muscle bands. Partial resolution of several bands was often obtained, and up to six bands could be distinguished in some cases, although additional bands of activity may well be present. As suggested by the antisera precipitation data, it is likely that at least one additional isozyme is present in these tissues. The electrophoretic pattern observed with brain extracts appeared to be less complex as shown in Fig. 4. At least four activity bands could be detected upon electrophoresis of brain extracts, none of which corresponded to either muscle or liver phosphofructokinase. Furthermore, the bands are more tightly grouped than the hybrids generated from muscle and liver phosphofructokinase. Shown on Fig. 4 is the activity band that remains in the supernatant solution after precipitation of enzyme activity by the addition of both antisera to muscle enzyme and antiserum to liver phosphofructokinase. A similar treatment of purified muscle and liver phosphofructokinase or of a mixture of muscle-liver hybrids leaves no detectable activity upon electrophoresis. It is presumed that the remaining activity band is a unique isozyme species. If the brain extract is treated only with antisera to muscle phosphofructokinase, the residual activity band shown in Fig. 4 is present along with faintly staining activity that moves more rapidly than the unique isozyme toward the anode. This material is assumed to be muscle-liver hybrids or hybrids of liver and the unique brain species because it is removed by treatment with antisera to the liver enzyme. Table IV shows the results of treating purified muscle, purified liver, and partially purified brain phosphofructokinases with the two antisera. Even with large excesses of the antisera, about 17% of the brain activity is not removed from solution. There may be a weak cross-reaction of the antisera with the unique brain isozyme. With 50 μl of muscle antiserum and 25 μl of liver antiserum, about 28% of the brain activity remains. Electrophoresis showed only one activity band. With four times as much antisera, again only one band of residual activity is observed and the activity has been reduced to 17%. That liver phosphofructokinase is actually present in small amounts in the brain is indicated by the removal of about 10% of the activity by very small amounts of liver antiserum (10 μl). An additional 10% is removed only by adding 100 μl of liver antiserum, presumably due to cross-reaction with other isozymes.
FIG. 4. Electrophoresis of brain extracts and antisera treated brain extracts. Electrophoresis for 3 hours at 340 volts and other conditions described under "Experimental Procedures" except that 3 mM ammonium sulfate was present in the electrophoresis buffer. Anode at the top; scale on the left indicates inches from origin. 1 and 2 are mixtures of muscle and liver phosphofructokinases. 4 is brain extract and 5 is brain extract after treatment of 0.1 ml of extract with 0.05 ml of antiserum to muscle enzyme and 0.025 ml of antiserum to liver enzyme followed by centrifugation at 18,000 X g for 20 min.

TABLE IV
Precipitation of brain phosphofructokinase (PFK) by antisera

Purified muscle and liver phosphofructokinase and a crude brain extract were used and antisera precipitations were carried out as described under "Experimental Procedures." The amount of enzyme used was 500 ~1 in each case diluted to 2 units per ml.

| Addition, antiserum to | Activity removed from |
|-----------------------|----------------------|
| Muscle PFK            | Liver PFK            |
| ~1                    | ~1                   |
| 50                    | 98                   | 32 | 67 |
| 200                   | 99                   | 50 | 83 |
|                        | 3                    | 96 | 14 |
|                        | 5                    | 98 | 20 |
|                        | 98                   | 97 | 72 |
| 50                    | 99                   | 99 | 83 |

Partial Purification of Brain Phosphofructokinase—Rabbit brain was homogenized with 3 volumes of the ice-cold extraction buffer described under "Experimental Procedures." To the extract, solid ammonium sulfate was slowly added to a final saturation of 0.6. After the suspension was stirred an additional 30 min, the sediment was collected by centrifugation at 15,000 X g and 4° and was dissolved to a volume equal to one-fourth that of the extract in a buffer consisting of 30 mM Tris-HCl, 150 mM ammonium sulfate, 2 mM EDTA, 1 mM dithiothreitol, 0.1 mM ATP, all at pH 8.0. The solution was placed in a 70° bath and stirred rapidly until the temperature of the solution reached 59°. The temperature was maintained within 1° of 59° for 3 min. The solution was rapidly cooled and the sediment was removed by centrifugation. The supernatant solution was again adjusted to 0.6 saturation with ammonium sulfate and the sediment was collected and dissolved in 30 mM Tris-HCl, 30 mM ammonium sulfate, 0.1 mM ATP, 2 mM EDTA, 1 mM dithiothreitol (pH 8.0) to a final volume equal to about one-twelfth that of the crude extract. The specific activity at this step was 1.45 units per mg and the recovery was 60%. An electrophoretic analysis indicated the same multiband pattern as is seen in Fig. 4.

Ion Exchange Chromatography of Muscle, Liver, and Brain Phosphofructokinases—The partially purified brain phosphofructokinase was subjected to chromatography on DEAE-Sephadex A-50 previously equilibrated with 30 mM Tris-HCl, 30 mM ammonium sulfate, 0.1 mM ATP, 2 mM EDTA, 1 mM dithiothreitol, all at pH 8.0. After the enzyme entered the column, the bed was further washed with the same buffer. The enzyme was eluted with a linear gradient from 30 mM ammonium sulfate to 500 mM ammonium sulfate in the Tris buffer. The elution profile is shown in Fig. 5. This figure represents a composite of three different chromatographic separations: purified muscle phosphofructokinase, purified liver phosphofructokinase, and partially purified brain enzyme. In each case, an identical column of DEAE-Sephadex (2.5 x 30 cm) was employed and
identical quantities of enzyme were applied (600 units). As predicted from the elecrotophoretic work, the muscle enzyme was bound least tightly to the anion exchange gel and it started to elute at an ammonium sulfate concentration of about 40 mM ammonium sulfate. The liver enzyme was spread over a large volume. This was noted on previous ion exchange purifications of the liver enzyme and had been attributed to the fact that the liver enzyme tends to form very large aggregates (8). When samples of the liver enzyme were removed from early and late fractions of the elution profile, no differences were noted in electrophoretic mobility or in reaction with the two types of antisera. On the other hand, phosphofructokinase activity from brain partially fractionated upon ion exchange chromatography, although clear separation of components was not achieved. Fractions from various portions of the activity peaks differed in their electrophoretic patterns and in their interactions with the two types of antisera. In no fraction was a single activity band observed, but several tubes showed only two bands. As expected, those fractions that eluted later moved more rapidly toward the anode than early eluting fractions. Enzyme activity that eluted near the front of the brain activity peak could react with the antibody to muscle enzyme but not the liver. For example, 75 to 80% of the activity from Fractions 26 to 29 (Fig. 5) could be precipitated by antibody to muscle enzyme under the conditions described in Table III. None of this activity was removed by 25 µl of the antisera to liver phosphofructokinase. On the other hand, Fractions 30 to 37 showed two faster moving activity bands upon electrophoresis, and only 42% of the activity could be precipitated by antisera to muscle phosphofructokinase and 18% by antisera to liver enzyme. When this fraction was subjected to electrophoresis after treatment by the two antisera, a single activity band was observed. Furthermore, in preliminary kinetic studies, this fraction was more sensitive to ATP inhibition than either the purified muscle or purified liver phosphofructokinases. Antisera precipitation carried out on fractions from the trailing edge of the brain activity peak (tube 40) indicated the presence of about 50% phosphofructokinase C and no comparison was made with phosphofructokinase B. More extensive kinetic analysis of sheep brain phosphofructokinase C was carried out by Lowry and Pascal (19), but the enzyme they examined had a specific activity of 18 units per mg and was isolated in a 4% yield. It is impossible to predict whether or not this preparation contained phosphofructokinase C and no comparison was made with phosphofructokinase activity from other sheep tissues. Kranowki and Matschinsky (21) prepared sheep brain phosphofructokinase by the method of Ling et al. (16) for muscle phosphofructokinase. The specific activity of their preparation was about 1 unit per mg. In this preparation as well, there is no way of knowing how much of the C isozyme was present. Kranowski and Matschinsky compared some properties of the brain preparation with those of a commercial rabbit muscle phosphofructokinase preparation and they observed that the brain enzyme was more sensitive to ATP and phosphocreatine inhibition. It is possible, however, that these are species-specific and not tissue-specific differences. A preliminary analysis of several partially resolved fractions shown in Fig. 5 indicated that the C isozyme is more sensitive to inhibition by ATP than either purified phosphofructokinase A or B. Further analysis must await the separation and identification of the various molecular forms of brain phosphofructokinase.

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