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ELECTROPHORETIC DIFFERENTIATION OF MULTIPLE FORMS OF PHOSPHOFRUCTOKINASE IN THE TISSUE FLUID OF THE RAT SKELETAL MUSCLE

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SUMMARY

1. Phosphofructokinase (ATP:D-fructose-6-phosphate phosphotransferase, EC 2.7.1.11) from rat skeletal muscle was prepared using a squeezing technique by which 40% of the muscle was obtained as a clear tissue fluid containing 60% of the total enzyme activity. Phosphofructokinase thus obtained was characterized by means of analytical zone electrophoresis and preparative disc electrophoresis in 3% polyacrylamide gels.

2. Only a small homogeneous fraction of the enzyme (containing 10% of phosphofructokinase activity) showed an electrophoretic migration to the anode, whereas the major part (90%) of the enzyme remained at the starting point as an aggregated form, with a molecular weight exceeding $1.5 \cdot 10^6$. This electrophoretic behaviour was found to be independent of the buffer system used, the reduction of the SH groups of the enzyme, or the protein concentration of the sample.

3. Homogenization of the muscle in buffers of different ionic strength during the fractional tissue extraction and homogenization of the muscle after squeezing caused a significant alteration of the electrophoretic behaviour of phosphofructokinase. In these cases only one enzymatically active fraction could be detected. This form of the enzyme did not differ electrophoretically from the low molecular form of phosphofructokinase obtained physically by the squeezing technique.

4. The multiple forms of phosphofructokinase from rat muscle, as demonstrated electrophoretically, may be explained by different aggregated forms of one and the same enzyme.

5. The physiological importance of these findings is discussed.

INTRODUCTION

Phosphofructokinase (ATP:D-fructose-6-phosphate phosphotransferase, EC 2.7.1.11) of mammalian muscle is localized exclusively within the cytoplasmatic cell compartment. The extractability of phosphofructokinase differs markedly from that of most of the glycolytic enzymes. During the fractional tissue extraction 60% of the total enzyme activity can be extracted using buffered sucrose solution and 40% only with a buffer of relative high ionic strength (0.1 M phosphate, pH 7.2, $I = 0.25$)[1].

These findings correlate well with the results published by MANSOUR *et al.*[2,3], who demonstrated that one part of phosphofructokinase can be extracted easily whereas a second fraction is extractable only in the presence of ATP and Mg^{2+}. These two forms of the enzyme can also be distinguished kinetically by means of different ATP inhibition. Two forms of phosphofructokinase from rabbit skeletal muscle differ in their sensitivity to acids[4]. ODEIDE *et al.*[5] have been able to crystallize two transient forms of the enzyme from rat muscle.

This paper presents a method of extracting phosphofructokinase from rat skeletal muscle. Denaturing agents are strictly avoided so that conclusions on the *in vivo* state of the enzyme may be drawn. For this purpose a special press has been developed which squeezes the tissue fluid from the muscle in amounts sufficient for the characterization of phosphofructokinase by preparative and analytical electrophoresis.

MATERIALS AND METHODS

The chemicals for the preparative electrophoresis (acrylamide, *N'*,*N'*-methylenebisacrylamide, tetramethylethylendiamine, ammonium persulphate) were obtained from Serva, Heidelberg, Germany. Amido black 10B and nitro blue–methosulphate were purchased from Sigma Chem. Corp., Philadelphia, Pa., U.S.A. Agarose was obtained from Behringwerke, Marburg, Germany. Substrates, enzymes and coenzymes were from Boehringer, Mannheim, Germany. All other chemicals were from E. Merck, Darmstadt, Germany.

Preparation of tissue fluid from rat skeletal muscle

Sprague–Dawley rats, starved for 48 h, were killed by cervical dislocation. A heavy pressure technique was used in order to obtain tissue fluid from muscle. The press consisted of a cylindric tube (length 25 cm, outer diameter 10 cm, inner diameter 4 cm), made from V_4A-steel. The outlet at the bottom of the press consisted of a small slot (40 mm long, 0.5 mm wide) which communicated with a buffer reservoir. Two nets, made from V_2A-steel (pore widths, 0.1 mm and 0.02 mm) were placed at the bottom of the press and the muscle, minced with scissors and wrapped in a small nylon bag, was layered onto them. Squeezing of the muscle was started in the pre-cooled press by joining the well-fitting V_4A-steel pestle of the press with a hydraulic pump (maximal weight 20 tons). The skeletal muscle was squeezed under standardized conditions ($p = 0.15$ ton/cm^2 (const.) per 3 min). The tissue fluid dropped directly into an equal volume of cold 100 mM phosphate buffer, pH 8.5, so as to obtain a pH of 7.2.

After centrifugation (16 000 $\times$ *g* for 4 min, Centrifuge 3200, Netheler und Hinz, Hamburg) the clear supernatant was either electrophoresized immediately or concentrated prior to electrophoresis by dialysis in special collodium bags (Membranfiltergesellschaft GmbH, Göttingen, Germany) against electrode buffer. The clear supernatant of the concentrated tissue fluid was again centrifuged (16 000 $\times$ *g* for 4 min) and then separated electrophoretically.

Electrophoresis

The preparative disc electrophoresis was performed in a vertical polyacrylamide gel (E.C. Apparatus Co., Philadelphia, U.S.A.).

The analytical zone electrophoresis was carried out on microscope slides (2.5 cm × 9 cm) in the horizontal position in a separating chamber constructed by us.

The electrophoretic runs carried out in the preparative chamber were done at a constant temperature (cooling system, −8°; buffer temperature, 8°) and a constant current (60 mA) for 13 — 16 h. Zonal electrophoresis on agarose was performed at 4° and at constant field strength of 5 V/cm for 3 h.

In the preparative system, a 3% separating gel and a 10% plug gel made from polyacrylamide were used. A sample gel was not used because of the very low polyacrylamide concentration in the separating gel.

The electrode buffer was 50 mM phosphate, pH 8.5. A 0.32 M triethanolamine–HCl buffer, pH 8.5, was used for the separating gels. Polymerisation of the polyacrylamide gel was achieved with ammonium persulphate (1.5 g/l). In order to prevent oxidation of the SH groups of phosphofructokinase, pre-electrophoreses were performed for 3 h, using cysteine–phosphate buffer (50 mM phosphate, 10 mM cysteine, pH 8.5). After the run the buffer was replaced by cold electrode buffer.

Histochemistry and controls

The position of the fractions containing phosphofructokinase activity was localized by staining the gels histochemically according to SIGEL AND PETTE[6], using the gel-layer technique[7], or incubating the separating gels directly with staining solutions.

Controls were run simultaneously in the absence of fructose 6-phosphate (Fru-6-*P*) and ATP. In this manner the unspecific reduction of tetrazolium salt by "nothing dehydrogenases"[8] could be demonstrated. The proteins were stained with Amido black 10B and destained with a mixture of methanol–glacial acetic acid–water (1:1:10, by vol.).

Phosphofructokinase test

Due to the time-dependent inactivation and the slow migration of phosphofructokinase in the electric field, the elution convection method[9], which removes crystalline enzymes quantitatively from the separating gel, could not be applied in the case of phosphofructokinase in tissue fluid.

Therefore the separating gels were cut into equal pieces (3 mm × 6 mm × 10 mm) immediately after the electrophoretic run. All gel segments were placed into a buffer containing 10 mM triethanolamine–HCl, 10 mM $MgSO_4$, 0.1 mM ATP, 0.1 mM β-mercaptoethanol, pH 8.0 (ref. 2), and homogenized by ultrasonication (Sonifier Branson Instruments Inc., Philadelphia, Pa., U.S.A.). More than 90% of the enzyme activity is set free by this procedure. After centrifugation (16 000 × *g* for 4 min), the phosphofructokinase activity was measured in the clear supernatants according to HOFER AND PETTE[10]. The enzyme activity is expressed in international units (U/ml). The recovery of phosphofructokinase was 82–86%.

Fractional extraction of phosphofructokinase from rat skeletal muscle

The fractional extraction of phosphofructokinase was carried out according to PETTE AND HOFER[1] and the different fractions were concentrated by dialysis in collodium bags as described.

RESULTS

A heavy pressure technique was used in order to obtain tissue fluid from rat skeletal muscle. Under optimal conditions (p (const.) = 0.15 ton/cm^2) about 40% of the muscle (wet weight) can be extracted as a clear tissue fluid.

Compared with the method of AMBERSON *et al.*[11] who used high speed centrifugation (40 000 rev./20 h) to obtain muscle extract, our procedure yields a higher amount of phosphofructokinase. 55–60% of the total phosphofructokinase activity is extracted by squeezing directly the tissue. This contrasts markedly with the centrifugation method which extracts only 10% of the enzyme activity.

Fig. 1 shows the electrophoretic differentiation of proteins and of phospho-

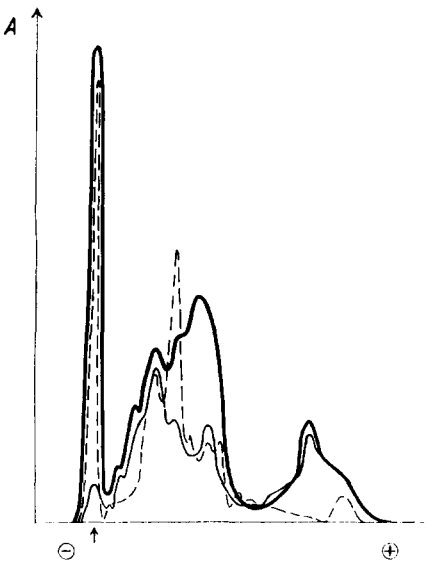

Fig. 1. Superimposed densitometric tracings of three parallel electrophoretic runs of phosphofructokinase from tissue fluid of rat skeletal muscle. Electrophoresis was performed in 3% polyacrylamide at 60 mA (const.) for 11 h. The point of origin is marked by the arrow. Electrophoretic migration is anodically from the left to the right. Protein concentration, 80.5 mg/ml. Sample volume, 0.04 ml each. Protein staining with Amido black 10B, densitometry at 405 nm (– – –). Histochemical staining of phosphofructokinase activity according to SIGEL AND PETTE[6], densitometry at 546 nm (———). Control staining (omitting ATP and Fru-6-*P*), densitometry at 546 nm (———).

fructokinase from muscle tissue fluid. Due to the rather low resolving power of a 3% polyacrylamide gel, the muscle proteins can only be separated into six protein-positive bands. In parallel runs omitting ATP and Fru-6-*P*, the substrates of phosphofructokinase, these bands also show a positive formazan reaction of variable intensity due to unspecific reductases.

Fig. 2 shows the result of an analytical electrophoresis of tissue fluid stained for phosphofructokinase. Four anodic migrating bands and one cathodic band, due to the electroendosmosis, can be distinguished. These results are very similar to those of the separation of lactate dehydrogenase isozymes, due to the presence of the "nothing dehydrogenases"[8].

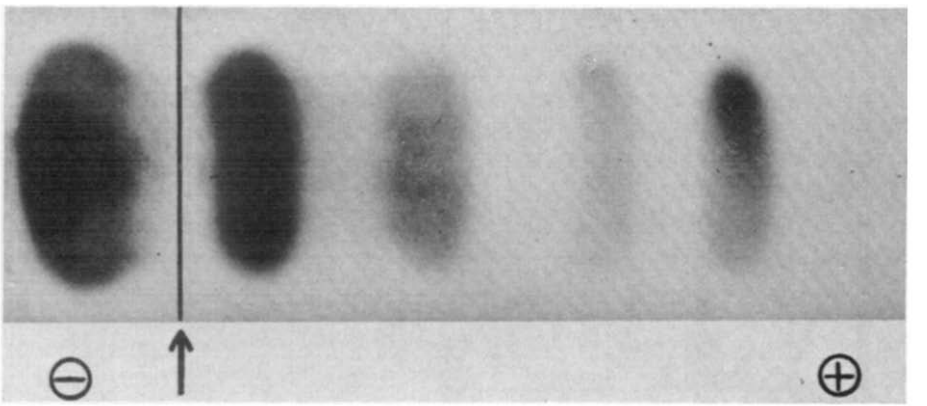

Fig. 2. Analytical zone electrophoresis on agarose of concentrated tissue fluid from rat skeletal muscle. Histochemical staining of phosphofructokinase according to ref. 6. The point of origin is marked by the arrow. Sample volume, 0.01 ml. Electrophoretic conditions: 3 h/5 V (const.) per cm.

Contrary to the above unspecific staining, the elution of phosphofructokinase from agarose shows (Fig. 3) that specific activity can be demonstrated only in the fraction with the slowest anodic mobility.

When compared with the results in Fig. 1, the behaviour of phosphofructokinase in the preparative electrophoresis becomes more interesting: the elution diagram of phosphofructokinase from tissue fluid of rat muscle after electrophoresis in 3% polyacrylamide can be seen in Fig. 4. Only a small amount of enzyme enters the separating gel: 90% of the enzyme activity remains at the origin while only 10% enters the gel.

The mobility of phosphofructokinase in the zonal electrophoresis is due mainly to the negative surplus charge at a given pH and no inhibition of the migration toward the anode is seen in our separating system (see Fig. 3); this indicates that the immobility of the main part of phosphofructokinase during preparative polyacrylamide

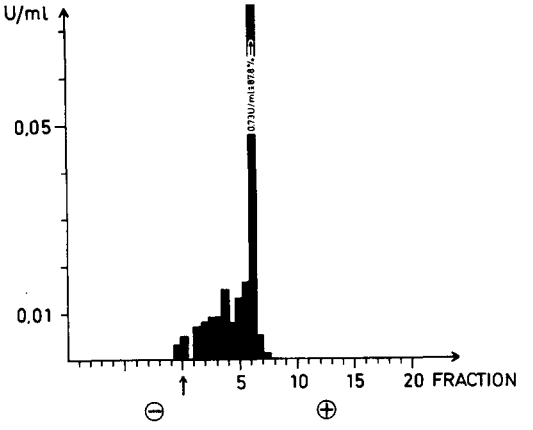

Fig. 3. Phosphofructokinase eluted from the electrophoretogram of Fig. 2. Enzyme activity is determined in the standardized optical test according to ref. 10. The point of origin is marked by the arrow.

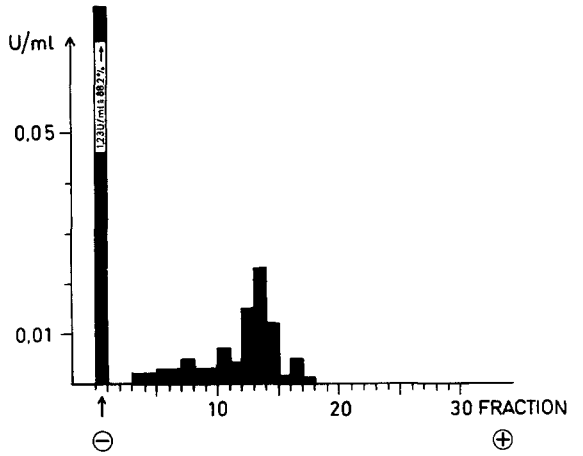

Fig. 4. Diagram of phosphofructokinase activity eluted from the preparative 3% polyacrylamide gel after electrophoretic separation of concentrated muscle tissue fluid of the rat. Protein concentration, 108 mg/ml. Sample volume, 0.06 ml. Electrophoretic conditions: 16 h/60 mA (const.).

electrophoresis is not due to electroneutrality but to the mechanical impossibility of entering the separating gel. This phenomenon can also be observed with glutamate dehydrogenase from bovine liver which is known to aggregate into polymers up to a molecular weight of $2.0 \cdot 10^6$ (ref. 12) and which is entirely excluded from a 3% polyacrylamide separating gel[13].

It is well known that purified and crystalline phosphofructokinase shows a tendency to form higher aggregates, mainly at high protein concentrations[14]. The possibility remained that the multiple forms of phosphofructokinase found in our system were an artefact produced by concentrating the sample prior to electrophoresis. To eliminate this factor, unconcentrated tissue fluid was subjected to disc gel electrophoresis under the same conditions. The results are presented in Fig. 5.

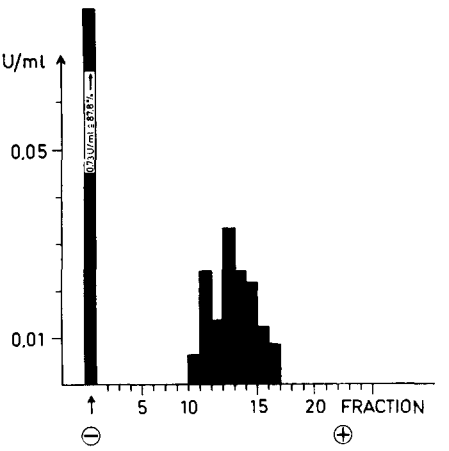

Fig. 5. Diagram of phosphofructokinase activity eluted from the preparative 3% polyacrylamide gel after electrophoretic separation of native tissue fluid from rat skeletal muscle. Protein concentration, 22.6 mg/ml. Sample volume, 0.60 ml. Separation conditions as in Fig. 4.

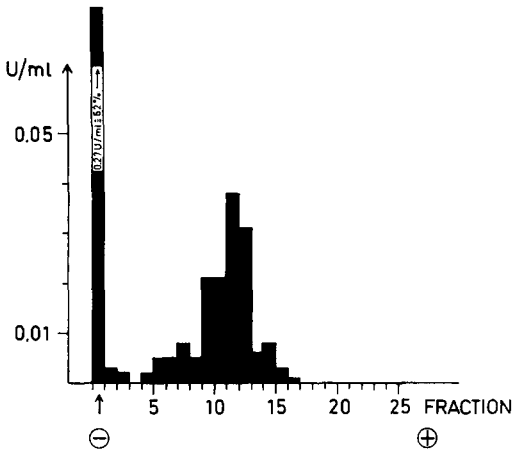

Fig. 6. Diagram of phosphofructokinase activity eluted from the preparative 3% polyacrylamide gel after electrophoretic separation of a sample diluted (1:10, w/v, with 0.1 M phosphate buffer, pH 7.6) prior to dialysis and electrophoresis. Protein concentration, 8.6 mg/ml. Sample volume, 0.06 ml. Electrophoretic conditions: 13 h/60 mA (const.).

Here, as in the case of concentrated tissue fluid, the maximum of enzyme activity could be detected at the point of origin, and the maximum peak of phosphofructokinase acticity within the separating gel was found at an identical position (Fraction 13).

The dilution of the tissue fluid with 0.1 M phosphate, pH 8.5 (ratio 1:10, v/v), did not significantly alter the distribution of enzymatic activity (see Fig. 6). At the origin, however, a reduction of the main peak of phosphofructokinase to about 60% of the total enzyme activity could be observed. This was probably caused by the phosphate ions. Almost no difference was found in the electrophoretic behaviour of the

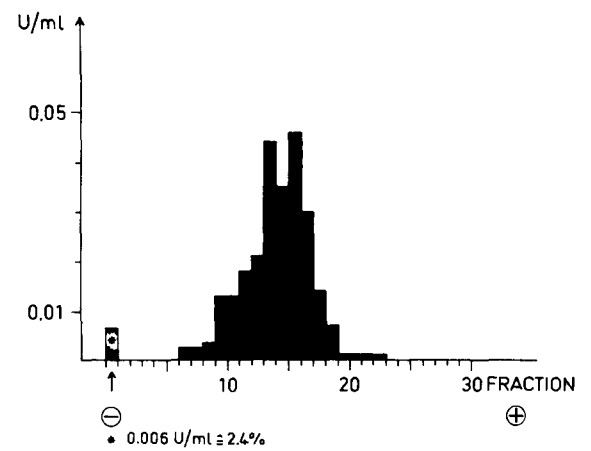

Fig. 7. Phosphofructokinase activity eluted from a 3% polyacrylamide gel. The sample was prepared by re-extracting the tissue after squeezing with 0.1 M phosphate, pH 7.6 (ratio 1:10, w/v), and dialyzing against electrode buffer prior to the electrophoretic separation. Separation conditions: 14 h/60 mA (const.).

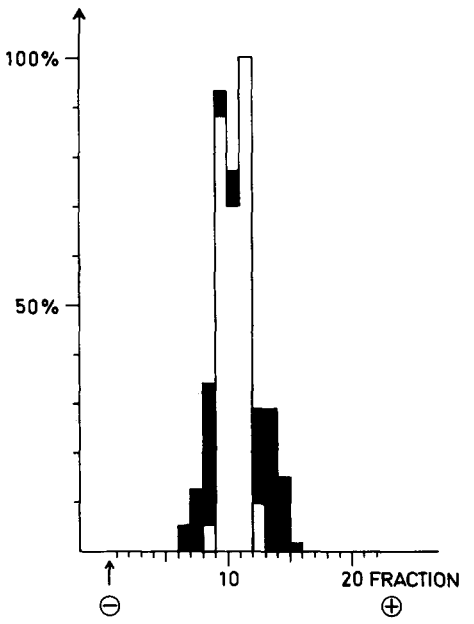

Fig. 8. Diagram of phosphofructokinase activity eluted from a 3% preparative polyacrylamide gel after electrophoretic separation. The enzyme was extracted from rat skeletal muscle by the method of fractional extraction[1]. White bars: Phosphofructokinase as extracted by contineously stirring rat muscle homogenate prepared with 0.3 M sucrose, 10 mM triethanolamine and 2 mM EDTA, pH 7.2. Protein concentration, 8.4 mg/ml. Black bars: Re-extraction of the same muscle with 0.1 M phosphate buffer, pH 7.2. Protein concentration, 1.65 mg/ml. The distribution of phosphofructokinase activity after elution from the separating gel is stated as relative percentage of the total enzyme activity.

minor part of phosphofructokinase, which entered the separating gel. This was comparable to the results obtained with concentrated and non-concentrated tissue fluid.

Striking differences in the electrophoretic behaviour of phosphofructokinase from tissue fluid of rat muscle were obtained when the tissue was extracted with phosphate buffer after being squeezed. The electrophoretic pattern (Fig. 7) shows that only traces of enzyme activity could be detected at the starting point, whereas most of the enzyme activity has entered the gel, forming a symmetrical double peak.

Both fractions of phosphofructokinase, as extracted from skeletal muscle according to PETTE AND HOFER[1], do not differ in their electrophoretic behaviour. Rather they have identical anodic mobility and form nearly identical peaks of activity, whereas no enzyme activity could be demonstrated at the point of origin (see Fig. 8).

DISCUSSION

To approach electrophoretically the problem of the physiological state of phosphofructokinase, we introduced a method of collecting tissue fluid from rat skeletal muscle by directly squeezing the tissue with a constant high pressure. Compared with other methods[11] of obtaining tissue fluid from muscle by physical procedures our technique improved the yield of phosphofructokinase 5–6-fold.

Preparative disc electrophoresis was performed to separate phosphofructokinase into two fractions of different activity and of different mobility. The major part (90%) of the enzyme was found to be immobile and could not enter the 3% polyacrylamide separating gel.

This phenomenon was due neither to the enzyme concentration nor to the pH of the separating system. Taken in combination with the electrophoretic behaviour of phosphofructokinase from tissue fluid in the analytical zone electrophoresis, this result could lead to the conclusion that the electrophoretic immobility of the major part of the enzyme must be due to the formation of higher aggregates, which are mechanically inhibited from entering the polyacrylamide separating gel.

Apart from this highly polymerized form of the enzyme, a markedly smaller fraction could always be detected within the separating gel. This homogeneous second form of phosphofructokinase shows an increase in activity when the pressed tissue is homogenized with phosphate buffer. In a similar way, the more aggregated form of phosphofructokinase can no longer be detected after fractional extraction of rat skeletal muscle.

It therefore seems reasonable to assume that the polymeric form of phosphofructokinase, as extracted physically, corresponds to the soluble cytoplasmic fraction of the enzyme. Homogenizing the tissue in buffered sucrose or in phosphate buffer produces only one oligomeric form of phosphofructokinase as shown by the identical mobilities in the polyacrylamide electrophoresis. This is probably attributable to a dissociation of protein–protein complexes.

The strong similarity between the electrophoretic behaviour of the minor fraction of phosphofructokinase from native tissue fluid and that of the enzyme extracted with phosphate buffer leads to the conclusion that the smaller band of phosphofructokinase could be due to a part of the enzyme which is bound to the outer mitochondrial membrane[15], and which can be eluted in the presence of phosphate ions.

The electrophoretic behaviour of purified and crystallized phosphofructokinase has already been investigated from mammalian muscle[2,14], but until now little has been known about the electrophoretic behaviour of the enzyme from tissue fluid.

SCOPES[16] demonstrated that electrophoresis of phosphofructokinase from the supernatant of homogenized muscle produced a slight homogeneous band showing little anodic mobility. These results agree well with our findings of sucrose- and phosphate-extracted phosphofructokinase (see Fig. 8).

When dealing with multiple forms of one enzyme, it is possible that the different fractions could be produced by methodical artefacts. These often occur when only histochemical staining of electrophoretograms is performed, due to unspecific tetrazolium reductases.

Moreover, different protein concentrations may cause multiple forms, especially of enzymes which tend to aggregate. This is not the case for phosphofructokinase from tissue fluid of rat muscle, since several experiments with native, concentrated and diluted tissue fluid have led to almost identical patterns of enzyme distribution in the separating gels. As demonstrated electrophoretically for catalase[17] and for trypsinogen[18], different oxidation states of the SH groups of the enzyme may easily produce "isozymes". These artefacts can be avoided by pre-electrophoresis with buffers containing SH reagents[19]. In our experiments a cysteine–phosphate buffer was used in order to remove oxidizing agents (*e.g.* ammonium persulphate).

Finally non-specific protein–buffer interactions can produce multiple fractions of homogeneous proteins. This phenomenon occurs when using borate buffers; phosphate and barbital buffers are largely inert[20]. In order to evade this possible source of error, several buffer systems of different ionic composition and pH have been tested. Using a continuous electrophoretic system of very low phosphate concentrations (10 mM phosphate, pH 7.6) instead of disc electrophoresis, very similar separations could be achieved.

Our results document the hypothesis that multiple forms of phosphofructokinase in the tissue fluid of rat skeletal muscle are not due to methodical artefacts but most probably reflect different states of aggregation of the enzyme representing a physiological phenomenon of the muscle cell.

From the diameter of the pores of the 3% polyacrylamide separating gel (width 20–40 Å), it can be calculated that the major fraction of phosphofructokinase which is excluded from the gel must exceed a molecular weight of $1.5 \cdot 10^6$. The transition of the oligomeric form of phosphofructokinase to the polymeric seems to depend on a phosphorylation system[3], regulated by negative and positive effectors of enzyme activity. This supports *in vitro* experiments which showed that phosphofructokinase is inhibited under physiological conditions and can only be regulated by its effectors[21,22].

The phenomenon of a reversible association–dissociation is known for other glycolytic enzymes. For example, hexokinase from frog muscle exists in different aggregated states[23]: a "light hexokinase" (mol.wt. 100 800) can be separated from a "heavy hexokinase" (mol.wt. 1960 000). These findings correlate with out results on phosphofructokinase. Other similarities concern the function of both enzymes as key enzymes of the glycolytic pathway, their intracellular localization as cytoplasmic and partially membrane bound enzymes, respectively, and their regulation by some identical effectors. The activation of hexokinase is known to be identical to the transition of the monomeric form into the polymeric[23]. The kinetic analysis of the phosphate dependent reactivation of phosphofructokinase shows this to be true only with limitations[22].

In summary, our results indicate that multiple electrophoretic forms of phosphofructokinase in the tissue fluid of rat skeletal muscle are not due to methodical artefacts but represent subunits of the enzyme at different states of aggregation. This probably reflects the physiological state of phosphofructokinase within the muscle cell as a regulated associating–dissociating enzyme system. Moreover, our findings confirm the results of ODEIDE *et al.*[5] who crystallized two transient forms of phosphofructokinase from rat muscle.

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