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# AVIAN FRUCTOSE-1,6-DIPHOSPHATASES

## I. PURIFICATION AND COMPARISON OF PHYSICAL AND IMMUNOLOGICAL PROPERTIES OF THE LIVER AND BREAST MUSCLE ENZYMES FROM CHICKEN (*GALLUS DOMESTICUS*)

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SUMMARY

1. Chicken muscle and chicken liver fructose-1,6-diphosphatases (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) have been purified. The enzymes were found to be homogeneous according to several criteria including purification to a constant specific activity, electrophoresis on cellulose acetate strips, absence of other glycolytic enzyme activities, and sedimentation velocity studies. The chicken liver enzyme was also tested for homogeneity by immunodiffusion on agar and by crystallization.

2. Comparisons of the two enzymes show that they have similar ultraviolet light absorption spectra but differ with respect to several other properties. The specific activity of the pure muscle enzyme is higher than that of the pure liver enzyme. Electrophoresis in several buffers and isoelectric point determinations (liver, 8.1 and muscle, 8.6) indicate that the avian enzymes differ in their electrostatic natures. The muscle enzyme ($s_{20,w}$ of 7.0) sediments at a slightly faster rate than the liver enzyme ($s_{20,w}$ of 6.8). Immunological analysis by double diffusion on agar and quantitative precipitin tests with anti-serum to chicken liver fructose-1,6-diphosphatase indicate that the liver and breast muscle enzymes differ immunologically. Kidney fructose-1,6-diphosphatase, in contrast, is immunologically similar to the liver enzyme. The anti-serum readily cross-reacts with other avian fructose-1,6-diphosphatases (turkey and budgerigar) but not with mammalian liver fructose-1,6-diphosphatases (rat and rabbit). The differences in properties of chicken liver and chicken breast muscle fructose-1,6-diphosphatases and immunological similarity of chicken liver and kidney fructose-1,6-diphosphatases suggest that there are at least two isozymic forms of avian fructose-1,6-diphosphatases.

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## INTRODUCTION

Fructose-1,6-diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) has been described as one of the key, rate-limiting, irreversible reactions of gluconeogenesis[1]. It is currently postulated that control of the activity of this enzyme by metabolite, allosteric, hormonal, and genetic effectors can act as one of the means of regulation of the rate of synthesis of carbohydrate from non-carbohydrate precursors[2].

Fructose-1,6-diphosphatase has been isolated from numerous biological sources[3–17]. Of particular interest are the fructose-1,6-diphosphatases isolated from mammalian liver[8–14], kidney[15,16] and skeletal muscle[17]. A comparison of the molecular and catalytic properties of liver, kidney, and muscle fructose-1,6-diphosphatases indicates a greater degree of similarity between the liver and kidney fructose-1,6-diphosphatases than between these two and muscle fructose-1,6-diphosphatase. Differences in electrophoretic mobilities, immunological cross-reactivity, and amino acid analysis but similarities in molecular weight and subunit structure indicate that there are two distinct isozymes of mammalian fructose-1,6-diphosphatase; one present in liver and kidney and the other present in skeletal muscle[17–31]. It has been postulated that each of these enzymes has a distinct physiological role. The liver enzyme is mainly involved in gluconeogenesis[22] while the muscle enzyme seems to be involved in regulating glycolysis[23–25].

Although a considerable amount of work has been carried out with mammalian liver and kidney fructose-1,6-diphosphatases very little is known about the role, function, and properties of avian tissue fructose-1,6-diphosphatases. The purpose of the present study has been the purification of fructose-1,6-diphosphatase from chicken liver and chicken breast muscle and the determination of several of the molecular properties of each.

## MATERIALS AND METHODS

### *Materials*

Chicken livers were obtained locally from a poultry processing company. Chicken breast muscle was obtained from growing hens taken from the University Poultry flock. Phosphocellulose, Tris, fructose 1,6-diphosphate, Sigma grade and grade II; Ponceau S; NADP; NADH; phenazine methosulfate and nitro blue tetrazolium chloride; were purchased from Sigma. Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49), glucosephosphate isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9), and rabbit muscle fructosediphosphate aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13) were purchased from Boehringer; dextran blue and Sephadex G-200 gel resin were from Pharmacia; Freund's complete adjuvant and Noble agar were from Difco; and Sepraphore III cellulose acetate strips and the barbital–Tris electrophoretic buffer mixture were from Gelman. DEAE(DE-52)-cellulose resin was from Whatman and carrier ampholyte was from LKB Produkter.

### *General methods*

Protein concentrations in the early purification steps were measured by the

method of Lowry *et al.*[26] using bovine serum albumin as a reference protein. For pure enzyme preparations, the absorbance at 280 nm was also used (mg of either liver or breast muscle fructose-1,6-diphosphatase per ml equals 1.36 × absorbance at 280 nm for a 1-cm light path).

*Enzyme assays*

Unless otherwise stated, all assays were run at 30 °C, the rate of change of either NADP or NADH concentration was measured at 340 nm. In all cases, a unit of enzyme activity is defined as that amount of enzyme catalyzing the disappearance of 1 μmole substrate per min under standard conditions.

For the routine assay of fructose-1,6-diphosphatase activity, the rate of formation of fructose 6-phosphate was measured spectrophotometrically by following the reduction of NADP in the presence of excess glucosephosphate isomerase and excess glucose-6-phosphate dehydrogenase. The reaction mixture (3.0 ml) contained 50 mM Tris, 1 mM EDTA (pH 7.5 at 30 °C), 10 mM $MgCl_2$, 0.15 mM NADP, 15 μg each of glucose-6-phosphate dehydrogenase and glucosephosphate isomerase, 0.0025 to 0.010 unit of fructose-1,6-diphosphatase and 0.15 mM FDP. The reaction was initiated with the addition of FDP or enzyme. The assays for the other enzymes have been described elsewhere[27–30].

*Zone electrophoresis*

Cellulose acetate electrophoresis was performed at 5 °C on 17 cm × 2.5 cm Sepraphore III strips in a Gelman electrophoresis chamber. The buffer systems used are described in the text.

*Immunological analysis*

Anti-serum to chicken liver fructose-1,6-diphosphatase was prepared in rabbits by subcutaneous injections of the chicken liver enzyme emulsified in Freund's complete adjuvant. Immunological analysis of fructose-1,6-diphosphatase with specific anti-serum was carried out using a modified two-dimensional gel diffusion (Ouchterlony) method as described by Campbell *et al.*[34].

*Isoelectric point*

The isoelectric points of the avian fructose-1,6-diphosphatases were determined by the method of Vesterberg and Svensson[35] with a LKB 8101 electrofocusing column and 8% carrier ampholyte "Ampholine" (pH 3–10 or 7–10). Liver fructose-1,6-diphosphatase (18.2 mg protein in 1.05 ml of 10 mM Tris, 1 mM EDTA, 100 mM KCl, 2 mM 2-mercaptoethanol, pH 7.5 at 10 °C) was added in equal portions to the gradient-forming mixing chamber and was initially focused with the pH 3–10 "Ampholine" for 90 h at 10 °C by applying a maximum of 3 W. After the analyses, the enzyme was refocalized for 60 h with the pH 7–10 "Ampholine".

The procedure for the determination of the isoelectric point of muscle fructose-1,6-diphosphatase was the same as that for liver fructose-1,6-diphosphatase except for the amounts of protein focused (2.4 mg for each determination) and the duration of the experiment (48 h).

*Sedimentation velocity analysis*

Samples for ultracentrifugation were prepared by diluting the concentrated enzyme with the buffer used for dialysis (10 mM Tris, 1 mM EDTA, 100 mM KCl, 2 mM 2-mercaptoethanol, pH 7.5 at 10 °C). Sedimentation velocity experiments were carried out with a Spinco Model E ultracentrifuge, using schlieren optics.

## RESULTS

*Purification of liver fructose-1,6-diphosphatase*

*Step 1. Extraction.* All steps were carried out at 0–3 °C unless otherwise stated. 300 g of frozen chicken livers were homogenized in 900 ml of 10 mM EDTA and 2 mM 2-mercaptoethanol (pH 7.5) at 5 °C. The homogenate was centrifuged at 25 000 × *g* for 30 min and the supernatant was passed through several layers of cheese cloth to remove fat particles.

*Step 2. Ammonium sulfate precipitation.* Solid ammonium sulfate was added to 45% saturation (250 g/200 ml) and the resultant precipitate was removed by centrifugation. Ammonium sulfate was then added to the clear supernatant to 65% (123 g additional ammonium sulfate/920 ml) and after standing overnight, the precipitate was collected by centrifugation. The pellet was dissolved in approximately 200 ml of Buffer I (20 mM Tris, 2 mM EDTA, 5 mM 2-mercaptoethanol, pH 7.5 at 10 °C) and was exhaustively dialyzed against the same buffer.

*Step 3. Phosphocellulose substrate elution chromatography.* The enzyme from the previous step was adsorbed onto a phosphocellulose column (39 cm × 3.7 cm) which had been previously equilibrated with Buffer 1. The column was washed with 2 l of Buffer 1 to remove unadsorbed proteins. Fructose-1,6-diphosphatase, fructosediphosphate aldolase, and pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) were then eluted (5 ml/min) with 600 ml of 2.5 mM FDP in the same buffer.

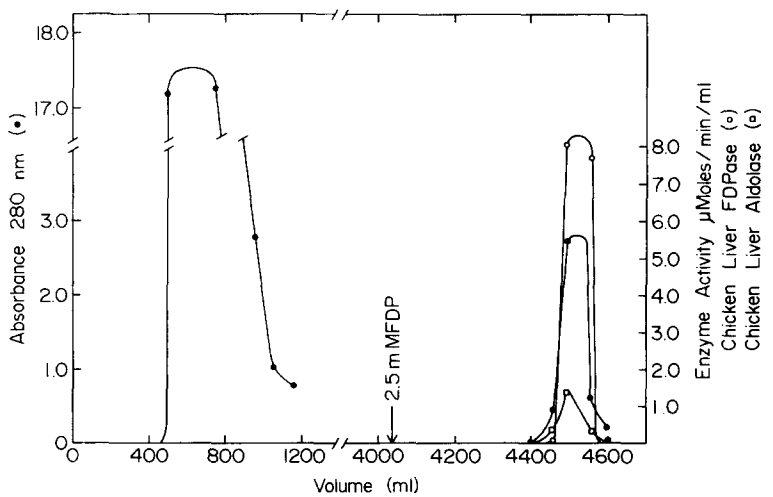


Fig. 1. Phosphocellulose chromatography of chicken liver fructose-1,6-diphosphatase (FDPase). FDP = fructose 1,6-diphosphate. For further details see text.

This separation was carried out at room temperature. A typical elution profile is given in Fig. 1.

The eluted enzyme was precipitated with 80% ammonium sulfate (71 g/126 ml), collected by centrifugation and dialyzed against 20 mM malonate, 2 mM EDTA, 3 mM 2-mercaptoethanol (pH 6.0).

*Steps 4 and 5. Ammonium sulfate crystallization.* After dialysis, the protein concentration was adjusted with the malonate buffer to approximately 8 mg/ml (the optimum concentration is from 6 to 12 mg/ml). Solid ammonium sulfate was added over 1 h to 49% (10.4 g/34 ml). Enzyme crystals started to form within a few hours and after standing 6 h were collected by centrifugation. The pellet was resuspended in a 50% ammonium sulfate–malonate buffer solution and was recentrifuged. The enzyme precipitate was then dissolved in the malonate buffer and was recrystallized. The amount of ammonium sulfate required to crystallize fructose-1,6-diphosphatase is affected by the relative purity of the enzyme and its concentration. Highly pure and more concentrated fructose-1,6-diphosphatase (10 to 15 mg/ml) preparations have been observed to crystallize at ammonium sulfate concentrations as low as 40%. The results of a typical purification are summarized in Table I.

TABLE I

PURIFICATION OF CHICKEN LIVER FRUCTOSE-1,6-DIPHOSPHATASE

| *Fraction* | *Total enzyme activity* | *Yield (%)* | *Specific activity (units/mg protein)* |
|---|---|---|---|
| 1 Extract | 3249 | 100 | 0.23 |
| 2 Ammonium sulfate precipitate (45–65%) | 1760 | 54 | 0.38 |
| 3 Phosphocellulose | 982 | 30 | 3.13 |
| 4 First crystallization | 913 | 28 | 13.6 |
| 5 Second crystallization (49% ammonium sulfate) | 631 | 19 | 13.7 |

*Purification of breast muscle fructose-1,6-diphosphatase*

*Step 1. Extraction.* 800 g of frozen chicken breast muscle were homogenized in 1800 ml of 100 mM Tris, 2 mM EDTA, 10 mM 2-mercaptoethanol (pH 8.0 at 10 °C). The homogenate was centrifuged at 25 000 × *g* for 30 min and the supernatant was passed through several layers of cheese cloth.

*Step 2. Ammonium sulfate precipitation.* The pH of the extract was adjusted to 8.5 at 10 °C with 1 M NaOH, with rapid stirring. Solid ammonium sulfate was added to 65% saturation (802 g/1866 ml). The resultant precipitate was removed by centrifugation and solid ammonium sulfate was then added to the supernatant to 80% saturation (214 g additional ammonium sulfate/2000 ml). After standing overnight the precipitate was collected by centrifugation, dissolved in approximately 100 ml of 50 mM Tris, 5 mM EDTA, 5 mM 2-mercaptoethanol (pH 8.0 at 10 °C) and exhaustively dialyzed against the same buffer (Buffer 2).

*Step 3. Phosphocellulose salt gradient chromatography.* The enzyme from the previous step was adsorbed onto a phosphocellulose column (26 cm × 2.8 cm) which had been previously equilibrated with Buffer 2. The column was washed with Buffer 2 to remove unadsorbed proteins and a linear salt gradient from 0–0.7 M KCl in

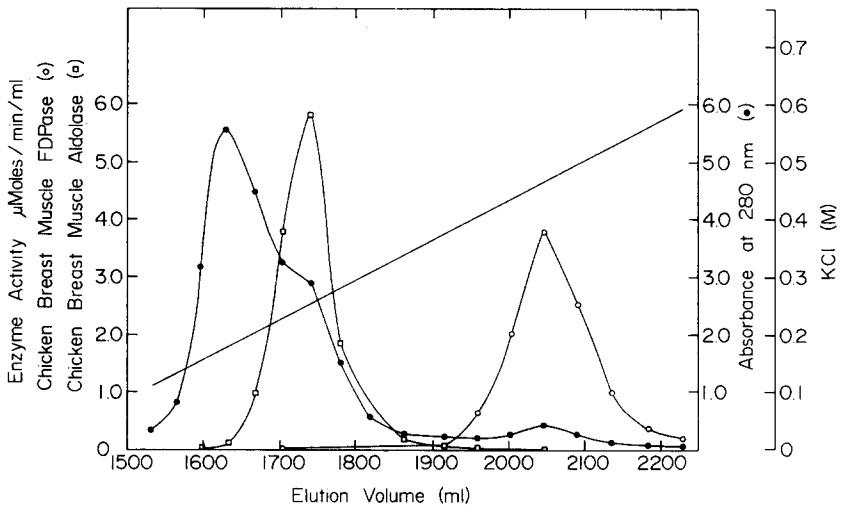


Fig. 2. Phosphocellulose chromatography of chicken breast muscle fructose-1,6-diphosphatase (FDPase); initial elution profile. See text for details.

Buffer 2 was then applied to the column. A typical elution profile is given in Fig. 2. The remaining adsorbed protein was removed from the column with 500 ml of 1 M KCl in Buffer 2 and the column was then re-equilibrated with 1 l of Buffer 2. Those fractions containing fructose-1,6-diphosphatase activity were pooled, diluted 1:3 with eluting buffer, and adsorbed again onto the phosphocellulose column. The column was washed with 500 ml of Buffer 2 to remove unadsorbed protein. A linear salt gradient (3 ml/min), similar to the one described above, was then applied to the column. A typical elution profile is given in Fig. 3. The eluted protein was precipitated with

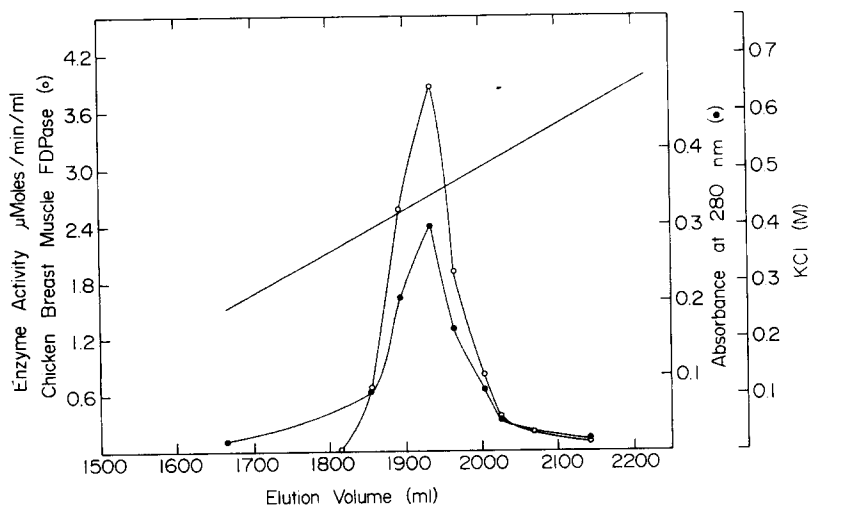


Fig. 3. Phosphocellulose chromatography of chicken breast muscle fructose-1,6-diphosphatase (FDPase); second elution profile. See text for details.

TABLE II

PURIFICATION OF CHICKEN BREAST MUSCLE FRUCTOSE-1,6-DIPHOSPHATASE

| *Fraction* | *Total enzyme activity* | *Yield (%)* | *Specific activity (units/mg protein)* |
|---|---|---|---|
| 1 Extract | 5721 | 100.0 | 0.17 |
| 2 Ammonium sulphate precipitate (65–80%) | 1196 | 20.9 | 0.64 |
| 3 Second phosphocellulose eluate | 466 | 8.2 | 23.4 |
| 4 Sephadex G-200 eluate | — | — | 22.5 |

85% ammonium sulfate (159 g/260 ml), collected by centrifugation, dissolved, and dialyzed against 50 mM Tris, 5 mM EDTA, 100 mM KCl, 2 mM 2-mercaptoethanol (pH 7.5 at 10 °C).

*Step 4. Sephadex G-200 chromatography.* Sephadex G-200 (40–120 nm) was prepared as described previously[27]. A portion of chicken breast muscle fructose-1,6-diphosphatase (6.95 mg/2.2 ml) from Step 3 was applied to a column (57.8 cm × 1.8 cm) previously equilibrated with the buffer in which the enzyme had been dialyzed. Elutions were carried out at 24 °C using a flow rate of approximately 4 ml/h. The results of a typical purification are summarized in Table II.

*Criteria of purity*

Chicken liver fructose-1,6-diphosphatase was tested for homogeneity by six methods: (a) purification to a constant specific activity, (b) cellulose acetate electrophoresis, (c) absence of other enzymatic activities, (d) ultracentrifugal analysis, (e) immunological analysis, and (f) crystallization of the enzyme. Muscle fructose-1,6-diphosphatase was tested for homogeneity using the first four methods.

Both enzymes retained constant specific activity during the final purification steps (Tables I and II). The pure enzymes appeared homogeneous when subjected to electrophoresis in four different buffers (Fig. 4). In all cases the proteins remained as a single band with no detectable contaminates other than a faint non-migrating protein. This protein which appears upon standing and is removed with centrifugation is denatured protein.

Often contaminants in an enzyme preparation are other enzymes which are, metabolically, closely related. Liver fructose-1,6-diphosphatase (Table I, Fraction 4) and muscle fructose-1,6-diphosphatase (Table II, Fraction 3) were assayed for several enzymes including fructosediphosphate aldolase, glucosephosphate isomerase, pyruvate kinase, glycerol-3-phosphate dehydrogenase (L-glycerol-3-phosphate: NAD oxidoreductase, EC 1.1.1.8), lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27), malic enzyme (L-malate: NADP oxidoreductase (decarboxylating), EC 1.1.1.40), glucose-6-phosphate dehydrogenase, glyceraldehydephosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating), EC 1.2.1.12) and triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketal-isomerase, EC 5.3.1.1). The percent contamination of the two fructose-1,6-diphosphatases preparations, when expressed on an activity basis, was less than 0.07% for all enzymes except for a 1% contamination of lactate dehydrogenase in muscle fruc-

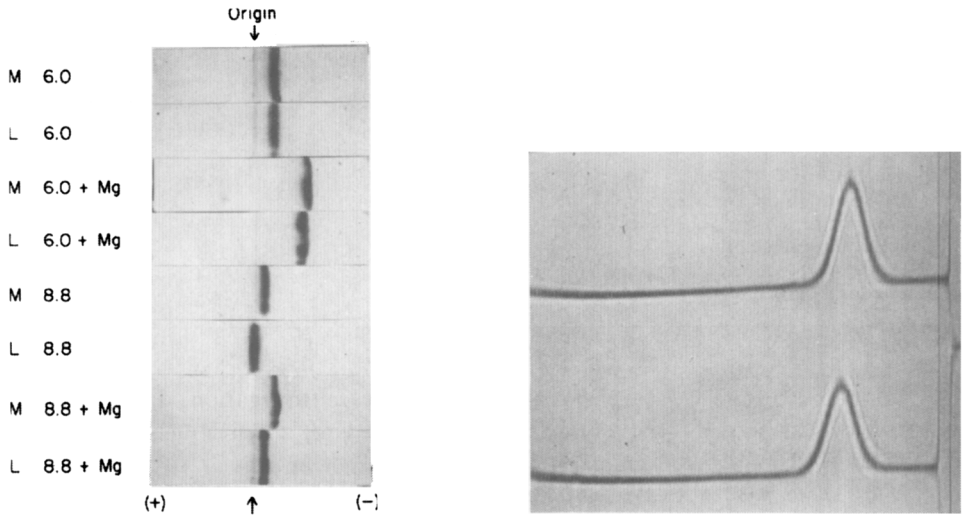


Fig. 4. Electrophoretic migration of pure chicken liver (L) and muscle (M) fructose-1,6-diphosphatases on cellulose acetate strips in the presence of various buffers. The 6.0 electrophoresis buffer contained 27 mM phosphate, 2.7 mM EDTA, 2 mM 2-mercaptoethanol, pH 6.0 while the 8.8 buffer was a Gelman–barbital–Tris salt mixture (17.8 g/l), 1 mM EDTA, 2 mM 2-mercaptoethanol, pH 8.8 Buffers 6.0 + Mg and 8.8 + Mg were the same as the above buffers except for the presence of 6 and 4 mM $MgCl_2$, respectively. Approximately 50 $\mu$g of protein were applied to the center of each strip. In all cases electrophoresis was carried out at 250 V for 80 min. The strips were stained with Ponceau S. See Materials and Methods for additional detail.

Fig. 5. Sedimentation velocity patterns of avian fructose-1,6-diphosphatases. Upper peak, chicken breast muscle fructose-1,6-diphosphatase (4.7 mg/ml); lower peak, chicken liver fructose-1,6-diphosphatase (4.7 mg/ml). The picture was taken 8 min after attaining top speed at a bar angle of 65°. Sedimentation is from right to left, See Materials and Methods for additional details.

tose-1,6-diphosphatase. On a protein basis the percent contamination of lactate dehydrogenase would be several fold less. This is due to the fact that lactate dehydrogenase has a much greater turnover rate than muscle fructose-1,6-diphosphatase[30].

The sedimentation velocity pattern of chicken liver (lower frame) and chicken muscle (upper frame) fructose-1,6-diphosphatase are shown in Fig. 5. In both cases only a single symmetrical peak developed, which suggested there was only one molecular species.

Anti-serum to chicken liver fructose-1,6-diphosphatase from rabbits was used in a modified Ouchterlony double diffusion method to determine the purity of the liver enzyme preparation. Only a single precipitin band appeared with crystalline liver fructose-1,6-diphosphatase and this line fused with a single precipitin line that developed with chicken liver extract (Fig. 6I). The presence of a single precipitin band with the extract suggests that the enzyme was pure. The crystalline form of the chicken liver enzyme is shown in Fig. 7.

*Electrophoretic mobilities*

An electrophoretic comparison of the two proteins in the presence of various buffers (Fig. 4) show that both enzymes have a higher rate of cationic migration at pH 6.0 as compared to pH 8.8. The addition of $MgCl_2$ to the electrophoretic buffer also enhances the rate of migration of both proteins especially at the lower pH. In

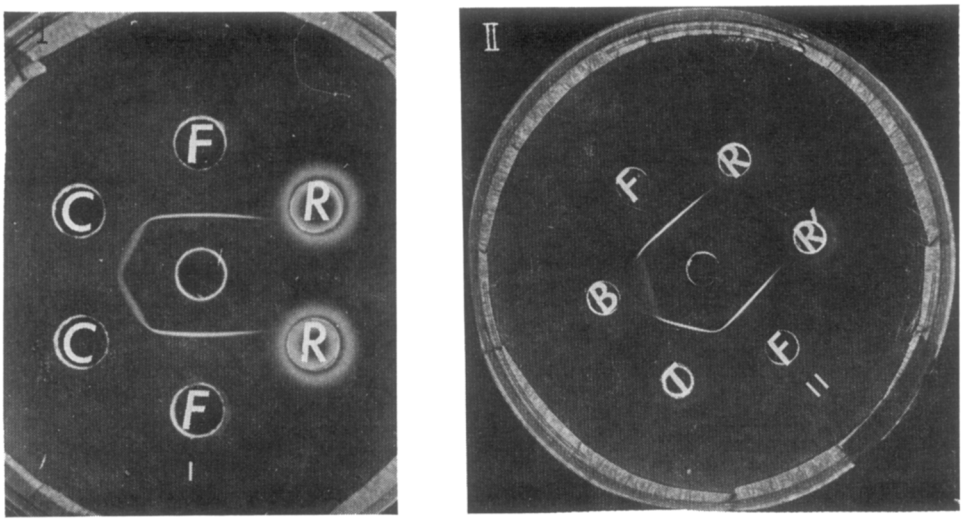


Fig. 6. Two dimensional immunodiffusion of various fructose-1,6-diphosphatases. The center wells contained chicken liver fructose-1,6-diphosphatase rabbit anti-serum. (I) Liver fructose-1,6-diphosphatases: F, crystalline chicken liver fructose-1,6-diphosphatase, 2.4 mg/ml (33 units/ml); C, chicken liver extract, 1.3 units/ml; R, rabbit liver extract, 1.1 units/ml. (II) Vertebrate liver fructose-1,6-diphosphatases: F, cystalline chicken liver fructose-1,6-diphosphatase, 1.35 mg/ml (18 units/ml); R, rabbit liver extract, 3.8 units/ml; R′, rat liver extract, 5.9 units/ml; B, budgerigar liver extract, 5.4 units/ml; and T, turkey liver extract, 8.2 units/ml. The halos around the center wells containing the extract are denatured protein. Tissue homogenates were prepared in 0.1 M Tris, 5 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 M KCl (pH 7.5 at 30 °C).

all cases the rate of migration of the muscle enzyme was slightly greater than that of the liver enzyme. In another experiment, a mixture of the two proteins was subjected to electrophoresis in the pH 8.8 buffer. Under these conditions two distinct but narrowly separated protein bands were observed, one being the liver enzyme, the other muscle fructose-1,6-diphosphatase.

*Isoelectric focusing*

When either fructose-1,6-diphosphatase was subjected to isoelectric focusing as described above only a single activity peak was obtained (Fig. 8). These peaks were coincident with single protein peaks. When determined over a pH 7–10 range, muscle fructose-1,6-diphosphatase showed an isoelectric point of 8.6 while liver fructose-1,6-diphosphatase had a value of 8.1 (Fig. 8). The values obtained when an ampholyte pH range of 3–10 was used were 8.6 for muscle and 8.0 for liver fructose-1,6-diphosphatases.

*Ultraviolet light absorption spectrum*

Pure muscle fructose-1,6-diphosphatase (2.0 mg/ml) and pure liver fructose-1,6-diphosphatase (2.5 mg/ml) in 20 mM Tris, 2 mM EDTA, (pH 7.5) do not absorb light in the visible region from 350–800 nm. The ultraviolet spectrum of both enzymes have a maximum at 279 nm with slight shoulders at 283 nm. The $A_{280\text{ nm}}/A_{260\text{ nm}}$ ratio is 2.44 for both enzymes.

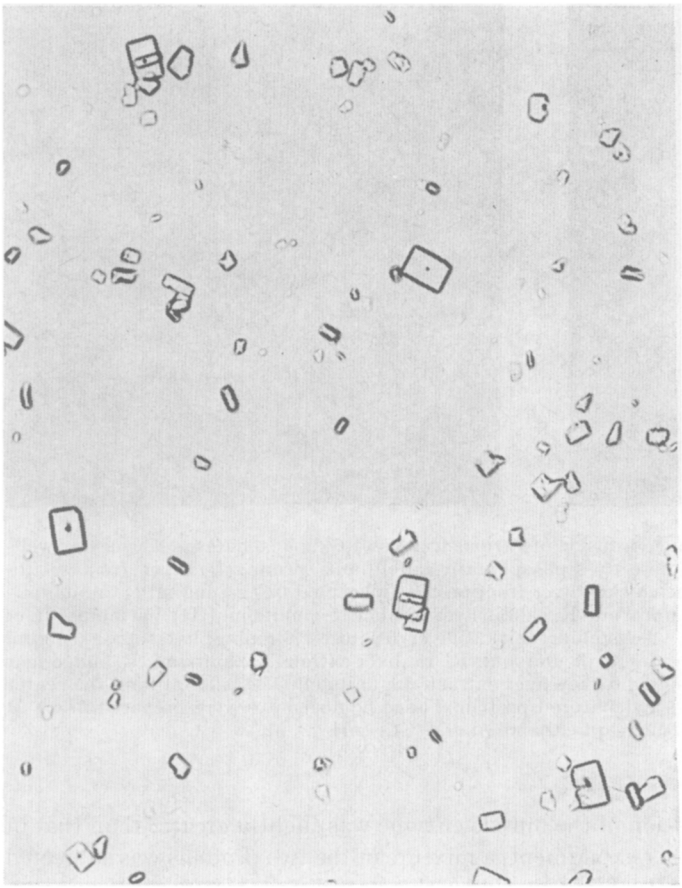

Fig. 7. Crystalline fructose-1,6-diphosphatase from chicken liver in an ammonium sulfate suspension (pH 6.0 at 7 °C). The pictures were taken with bright-field objectives on a Zeiss photomicroscope under 63-fold magnification.

*Sedimentation velocity*

The two enzymes sedimented as a single symmetrical peak (Fig. 5) with a $s_{20,w}$ value of 7.0 S for the muscle enzyme and 6.8 S for the liver enzyme.

*Immunochemical analyses*

Immunodiffusion results show a strong reaction between the antiserum and the pure enzyme and the enzyme present in chicken liver extract (Fig. 6I). Kidney extract fructose-1,6-diphosphatase also forms a continuous precipitin band with liver fructose-1,6-diphosphatase (Fig. 9I). No cross-reaction, however, was observed with the fructose-1,6-diphosphatases from muscle, brain or heart. The failure of these tissues to show a cross-reaction may be due, in part, to low levels of fructose-1,6-diphosphatase in the tissue extracts. In another experiment (Fig. 9II) a much higher concentration of muscle fructose-1,6-diphosphatase was used; under these conditions a weak cross-reaction between concentrated pure muscle fructose-1,6-diphosphatase and the antiserum was observed. The muscle precipitin band, which showed spur

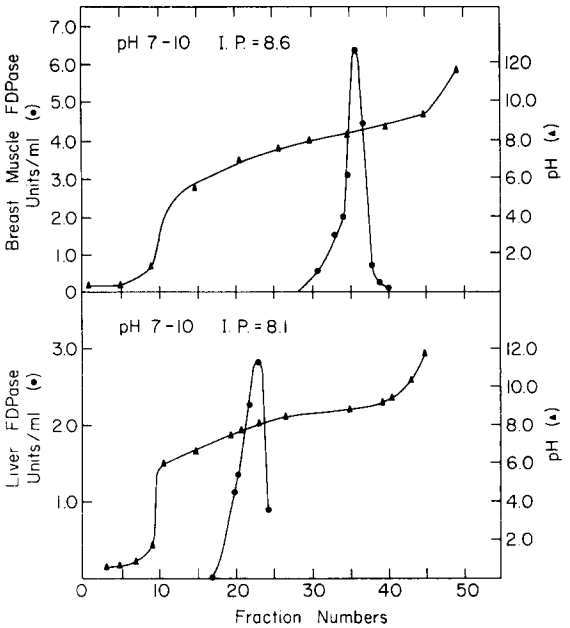


Fig. 8. Profiles of isolectric point (I.P.) determinations of muscle and liver fructose-1,6-diphosphatases. Electrofocusing of the enzymes was carried out as described in Materials and Methods.

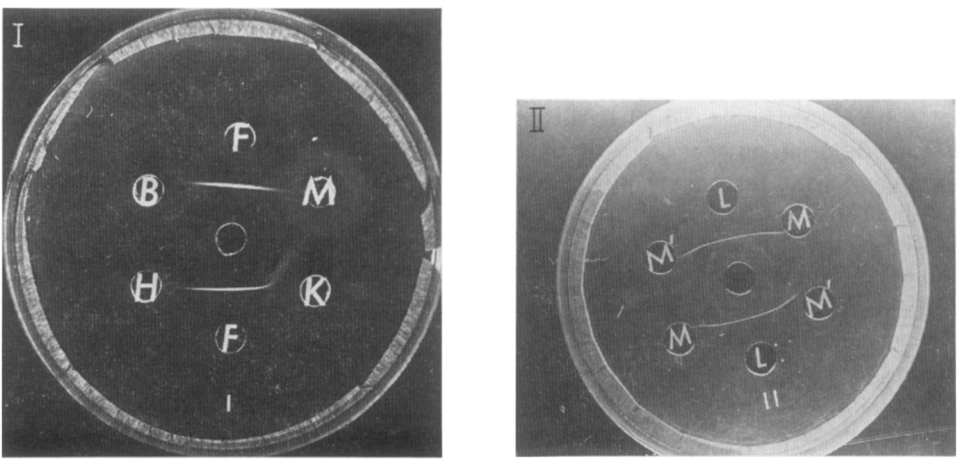


Fig. 9. Double diffusion analysis of chicken tissue fructose-1,6-diphosphatases. (I) F, pure liver fructose-1,6-diphosphatase, 1.35 mg/ml (18 units/ml); K, kidney extract, 3.1 units/ml; M, breast muscle extract, 1.7 units/ml; B, brain extract, 0.45 units/ml; and H, heart extract, 0.03 units/ml. (II) L, pure liver fructose-1,6-diphosphatase, 1.2 mg/ml; M and M', pure breast muscle fructose-1,6-diphosphatase, 4.7 and 2.3 mg/ml, respectively. The center well contained chicken liver fructose-1,6-diphosphatase rabbit antiserum. See Fig. 6 and Materials and Methods for additional detail.

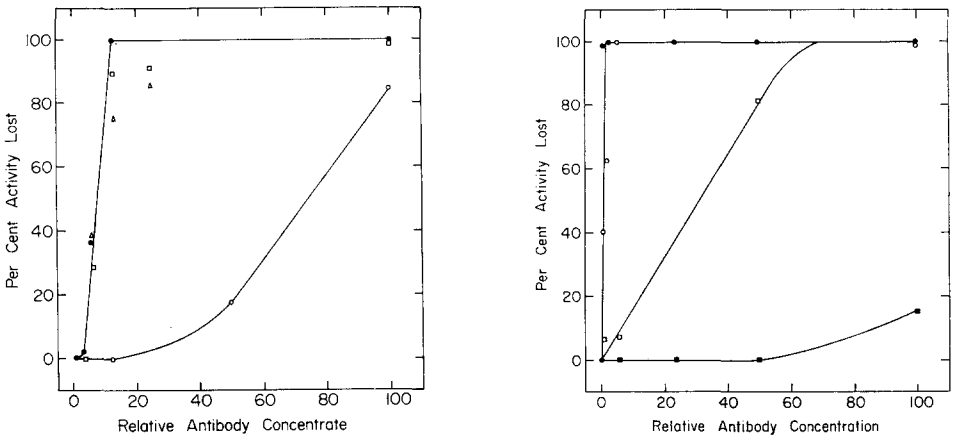


Fig. 10. Precipitation of crystalline chicken liver fructose-1,6-diphosphatase (●) and the fructose-1,6-diphosphatases from extracts of chicken liver (□), kidney (△), and breast muscle (○) with anti-serum to chicken liver fructose-1,6-diphosphatase. Eight doubling dilutions of anti-serum were prepared in 50 mM Tris, 5 mM EDTA, 0.14 M NaCl, 1% bovine serum albumin (pH 7.5 at 10 °C). To each 0.5 ml of diluted antibody, 0.1 ml of crystalline chicken liver enzyme (2.1 units/ml) or tissue extract (2.1 units/ml) was added. After incubation at 30 °C (for 30 min and 2 °C for 16 h, the precipitates were removed by centrifugation at 50 000 × *g* for 20 min and fructose-1,6-diphosphatase was assayed in each filtrate.

Fig. 11. Precipitation of crystalline chicken liver fructose-1,6-diphosphatase (○) and the liver fructose-1,6-diphosphatases of turkey (●), budgerigar (□) and rabbit (■) with anti-serum to chicken liver fructose-1,6-diphosphatase. Conditions were as described in Fig. 10 except that enzyme concentrations were 2.5 units/ml.

formation, was not continuous with the liver enzyme. The quantitative precipitin patterns of pure liver fructose-1,6-diphosphatase and those of chicken liver and kidney extracts were similar (Fig. 10). Low concentrations of anti-liver fructose-1,6-diphosphatase completely precipitated the enzyme from all three samples. The antiserum, however, had much less effect on muscle fructose-1,6-diphosphatase.

Liver fructose-1,6-diphosphatases from several species were also compared. Double diffusion on agar show that there is a good cross-reaction between the antiserum and crystalline chicken liver fructose-1,6-diphosphatase and the liver fructose-1,6-diphosphatases of budgerigar and turkey (Fig. 6II). The continuity between the precipitin bands of chicken liver fructose-1,6-diphosphatase and turkey liver extract fructose-1,6-diphosphatase suggested a fairly high degree of similarity between the two enzymes. However, spur formation between the precipitin bands of budgerigar liver extract fructose-1,6-diphosphatase and chicken and turkey liver fructose-1,6-diphosphatases suggest that budgerigar liver fructose-1,6-diphosphatase is immunologically different from the fructose-1,6-diphosphatases of chicken and turkey livers. There was, however, little or no cross-reaction with the liver fructose-1,6-diphosphatases of rat and rabbit. Quantitative precipitation results (Fig. 11) are consistent with the above results.

## DISCUSSION

In the current research the employment of fructose 1,6-diphosphate to speci-

fically elute fructose-1,6-diphosphatase, fructosediphosphate aldolase and pyruvate kinase from a phosphocellulose column followed by the selective crystallization of fructose-1,6-diphosphatase are the two steps which greatly facilitate the purification of chicken liver fructose-1,6-diphosphatase. In the purification of chicken muscle fructose-1,6-diphosphatase, substrate elution was not employed. The important steps in this isolation are ammonium sulfate fractionation followed by two successive gradient elutions with KCl from a phosphocellulose column.

In this study a considerable loss in activity of the muscle enzyme and to a lesser extent the liver enzyme occurred during the isolation procedure. Both enzymes, however, were stable for several months when stored in an ammonium sulfate suspension. The stabilities of both enzymes are also influenced by various cofactors, pH and temperature[36].

The isoelectric points of the two fructose-1,6-diphosphatases as determined by isoelectric focusing were 8.1 for the liver enzyme and 8.6 for the muscle enzyme. These values are consistent with the zone electrophoresis results but are considerably different to the estimated values reported for various mammalian fructose-1,6-diphosphatases[16,20]. Mendicino *et al.*[16] reported an approximate isoelectric point of 5.9 for swine kidney fructose-1,6-diphosphatase. Enser *et al.*[20] have suggested that the isoelectric point of rabbit muscle fructose-1,6-diphosphatase is close to pH 6.5 and that the isoelectric values for the rabbit liver and rabbit kidney fructose-1,6-diphosphatases were the same but higher than 6.5. Cellulose acetate results suggest that the isoelectric values of the two proteins are influenced by the composition of the electrophoretic buffer. The addition of $MgCl_2$, which is a cofactor for the enzyme, increases the rate of migration of both proteins. Fructose 1,6-diphosphate and AMP, which also interact with the two fructose-1,6-diphosphatases, should alter the electrostatic charge associated with the enzymes; as a result, electrophoretic mobilities would be affected. Substrate elution of fructose-1,6-diphosphatases from a phosphocellulose column support the proposal that the electrostatic charge of the two proteins can be altered by substrate.

The different sedimentation values of chicken muscle (7.0 S) and chicken liver (6.8 S) fructose-1,6-diphosphatases suggests that these two proteins may differ in molecular properties. A more detailed comparison of these properties will be published elsewhere. The reported sedimentation coefficients for mammalian fructose-1,6-diphosphatases are: 7.2 S rabbit liver fructose-1,6-diphosphatase[11,37]; 7.0 S rabbit muscle fructose-1,6-diphosphatase[17]; and 7.5 S swine kidney fructose-1,6-diphosphatase[16].

The immunological studies show that chicken liver and chicken muscle fructose-1,6-diphosphatases are different while chicken liver and chicken kidney fructose-1,6-diphosphatases are similar. Similar results were reported for the fructose-1,6-diphosphatase isozymes of the rabbit by Enser *et al.*[20]. Cross-reactions between the antiserum and various liver fructose-1,6-diphosphatases show that there are marked species differences. The mammalian enzymes (rabbit and rat) do not cross-react with the anti-serum while certain of the avian enzymes (chicken, turkey and budgerigar) are readily precipitated. The immunodiffusion and precipitin results also indicate that the budgerigar fructose-1,6-diphosphatase is quite different than that of chicken liver fructose-1,6-diphosphatase while turkey liver fructose-1,6-diphosphatase is similar to chicken liver fructose-1,6-diphosphatase. A more sensitive immuno-

chemical test, such as the microcomplement fixation test[38], would provide a more definitive comparison for certain of the enzymes.

The results from this paper suggest that there are at least two isozymic forms of avian fructose-1,6-diphosphatases. One form can be considered to be located in the liver and kidney of the chicken and the other form in chicken breast muscle.

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