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Avian Fructose 1,6-Diphosphatases: Purification and Several Physical Properties of Chicken Liver and Breast Muscle Fructose 1,6-Diphosphatases

by

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Abstract

Fructose 1,6-diphosphatase (FDPase) (D-Fructose 1,6-diphosphate 1-phosphorylase, EC 3.1.3.11) was purified from chicken liver and chicken breast muscle.

The enzymes were found to be homogeneous according to the following criteria of purity: purification to a constant specific activity, electrophoresis on cellulose acetate strips, absence of other glycolytic enzyme activities, and sedimentation velocity studies. Chicken liver FDPase was also tested for homogeneity by immunodiffusion on agar and by crystallization of the enzyme.

Electrophoresis at pH 8.8 and isoelectric point determinations indicate that the avian isozymes differ in their electrostatic natures.

Immunological analysis by double diffusion on agar and quantitative precipitin tests with anti-serum to chicken liver FDPase indicate that the liver and breast muscle FDPases differ immunologically. On the other hand, immunological analysis show that pure FDPases of chicken liver and the FDPase of chicken kidney extracts are immunologically similar.

Amino acid analysis of the two enzymes show that the two enzymes differ significantly in the molar concentrations of the majority of their constitutive amino acids.

The molecular weights of the chicken liver and chicken breast muscle FDPases were determined by

chromatography on Sephadex G-200. Chicken liver FDPase had a molecular weight of 134,900 ± 4400 while that of breast muscle FDPase was 152,050 ± 500. In addition, sedimentation coefficient studies yielded a molecular weight of 143,300 for chicken liver FDPase.

The differences in molecular properties of the chicken liver and breast muscle FDPases and the immunological similarity of the chicken liver and kidney FDPases suggest that there are two isozymic forms of avian FDPases. 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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List of Abbreviations

AMP	adenosine 5'-monophosphate
CoA	coenzyme A
DTNB	5,5'-dithiobis-(2-nitrilo-benzoic acid)
$D_{20,w}$	diffusion coefficient
EDTA	(ethylene dinitrilo) tetraacetic acid
EtOH	ethanol
FDP	fructose 1,6-diphosphate
FDPase	fructose 1,6-diphosphatase
Km	dissociation constant
NAD	nicotinamide adenine dinuclectide
$NADH_2$	nicotinamide adenine dinucleotide, reduced form
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
SDP	sedoheptulose 1,6-diphosphate
-SH	sulfhydryl group
$S_{20,w}$	sedimentation coefficient
Tris	tris (hydroxymethyl) aminomethane

Introduction

Fructose 1,6-diphosphatase (FDPase) (D-Fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) catalyzes the reaction D-fructose 1,6-diphosphate + H_2O → D-fructose 6-phosphate + orthophosphate. This has been described as one of the key, rate-limiting, irreversible reactions of gluconeogenesis (92). 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.

FDPase has been isolated from numerous biological sources (5-8, 17, 18, 22, 23, 37, 45, 48, 71-77, 80, 85). Of particular interest is the FDPases isolated from mammalian liver (8, 71), kidney (17, 37, 45), and skeletal muscle (18). A comparison of the data from studies performed on the FDPases of these tissues indicates differences in catalytic, allosteric, and molecular properties. A comparison of the molecular properties of liver, kidney, and muscle FDPases indicates a greater degree of similarity between the liver and kidney FDPases than between these two and the muscle FDPase. 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 FDPase; one present

in liver and kidney and the other present in skeletal muscle. It has been postulated that each of these enzymes has a distinct physiological role. The liver enzyme is mainly involved in gluconeogenesis (92) while the muscle enzyme seems to be involved in regulating glycolysis (30, 53, 54).

Although a considerable amount of work has been carried out with mammalian FDPases, very little is known about the role, function, and properties of the FDPases of avain tissues. The purpose of the present study has been the purification of FDPase from chicken liver and chicken breast muscle and the determination of several of the molecular properties of each. A comparison of these porperties was made in order to estimate the degree of divergence between these enzymes.

Review of the Literature

A specific fructose 1,6-diphosphatase (FDPase) (EC 3.1.3.11) that catalyzes the reaction: D-fructose 1,6-diphosphate + H_2O → D-fructose 6-phosphate + inorganic phosphate was originally discovered and isolated from rabbit liver by Gomori (23) in 1943. Until the symposium, in 1961, on the role of FDPase in gluconeogenesis (44), relatively little interest was taken in this enzyme. Since that time, however, FDPase has been the subject of numerous investigations into its purification, its chemical and physical properties, its role in carbohydrate metabolism and its regulation.

A. Role and General Properties of Fructose 1,6-Diphosphatase

Generally speaking, it has been found that FDPase, in its mediation of the above irreversible reaction, plays a key role in gluconeogenesis by acting as one of the rate-limiting enzymes of the overall process (93). Hence, regulation of gluconeogenesis could be acheived by controlling the activity of this enzyme. FDPase has been isolated from unicellular (5, 22, 48, 73-76, 85), plant (72, 80), poikilotherm liver (6, 7), amphibian muscle (77), mammalian liver (8, 23, 71), mammalian kidney (17, 37, 45), and mammalian muscle (18) sources. It has, generally been found to be subject to allosteric inhibition by AMP and high substrate concentrations; has a high affinity

for its substrate; has an absolute requirement for a divalent metal such as Mg^{2+} or Mn^{2+}, and is active in the physiological pH range in the presence of a chelating agent such as EDTA. Its activity is also affected by interactions of pH, temperature, and the above effectors. Equally important is its regulation by nutritional, hormonal, and genetic control mechanisms (93).

A knowledge of the comparative properties of FDPase from various tissues and species has enabled researchers to surmise a more precise role of the enzyme in the regulation of carbohydrate metabolism. In some cases (5, 6, 7), these metabolic roles have been correlated to evolutionary adaptations which the organism had to make in order to make efficient use of its environment.

B. Properties of Fructose 1,6-Diphosphatases from Various Mammalian Tissue Sources

During the past decade, several independent groups of researchers have isolated and studied the catalytic, allosteric, and molecular properties of the FDPases of various mammalian tissues. These groups have taken special interest in the isolation and determination of the characteristics of FDPases from mammalian liver, kidney, and skeletal muscle sources. The most extensive studies on mammalian FDPases have been those of Horecker, Pontremoli, Pogell, and associates. These researchers

have been mainly concerned with the properties of purified rabbit liver FDPase (57-64, 66-71). Recently they have purified FDPase from rabbit skeletal muscle and have carried out determinations of some of its kinetic and molecular properties (18). Bonsignore et al. (8), Marcus (37), Mendicino et al. (45), and Enser et al. (17) have also carried out independent studies on purified rat liver, swine kidney, and rabbit kidney FDPases.

A Comparison of the Catalytic Properties

It is a well understood fact that the activity exhibited by any given enzyme is strongly affected by the conditions under which the assay is carried out. A given effect may be considered to be due to the catalytic properties inherent to the given enzyme. In the case of mammalian FDPases, variations in activity under varying conditions may be due to the inherent substrate specificity, dissociation constants (Km), substrate inhibition, requirement for cations, and pH optimum of the enzymes. A comparison of the mammalian liver, kidney, and muscle FDPases on the basis of these catalytic properties may give an insight into the differences and similarities between these enzymes.

Pontremoli et al. (71) found that purified rabbit liver FDPase was capable of catalyzing the hydrolysis of sedoheptulose 1,7-diphosphate (SDP) in addition to being able to catalyze the hydrolysis of fructose 1,6-

diphosphate (FDP). The rate of hydrolysis of FDP was about 1.6 times that of SDP. In earlier experiments with purified rat liver FDPase, Bonsignore et al. (8) found that the Km for the reaction with FDP was approximately 25-fold lower than that for SDP while the ratios of the activity of the enzyme with the two substrates always approximated unity. Fernando et al. (18) also found that rabbit muscle FDPase catalyzes the hydrolysis of the substrates at approximately the same rate. On the basis of these studies and the fact that the physiological role of the hydrolysis of SDP has not been established (65), it may be tentatively said that FDP constitutes the major substrate of the liver enzyme. It is not apparent from the literature available whether or not this holds true for mammalian muscle and kidney FDPases. However, speculation deems that FDP is probably the major substrate of these FDPases as well.

Mammalian FDPases have been found to be active at low substrate concentrations. Sato and Tsuiki (79) reported that, at pH 7.4 in the presence of 10mM Mg^{2+}, the FDPases of rat liver, kidney, and muscle exhibited Km's of 2.1, 2.5, and 4.0×10^{-6} M, respectively. Bonsignore et al. (8) reported a Km of 1.2×10^{-5} M at pH 9.0 at optimal Mn^{2+} concentrations for rat liver. Pontremoli et al. (69) reported that, in the presence of either Mg^{2+} or Mn^{2+} and at pH 7.5, rabbit liver FDPase exhibits a Km of less than 1×10^{-6} M.

However, they found that, at pH 9.1, rabbit liver FDPase exhibited Km's of 4.3×10^{-6} M in the presence of Mg^{2+} and 2.6×10^{-6} M in the presence of Mn^{2+}. Fernando et al. (18) reported a Km that appears to be in the order of 10^{-6} M for muscle FDPase at neutral pH in the presence of 5 mM Mg^{2+}, which was in agreement with the values reported by Krebs and Woodford (30). In the presence of 0.5 mM Mn^{2+} and at pH 9.3 the enzyme exhibited a Km of 3.5×10^{-6} M. Thus, it seems that mammalian FDPases are activate at approximately the same substrate concentrations, irregardless of pH or cation.

It is of interest to note that mammalian FDPases were subject to inhibition by excess substrate concentrations (18, 30, 50, 79). Sato and Tsuiki (79) reported that concentrations of FDP higher than 5×10^{-5} M inhibited rat liver, kidney, and skeletal muscle FDPases to similar extents. Nakashima et al. (50) reported that, for rabbit liver FDPase, at 1×10^{-3} M FDP and in the presence of either Mn^{2+} or Mg^{2+}, the rate of hydrolysis is only about 50% of the optimum rate. At higher concentrations the enzyme was inhibited more than 80%. In contrast, Fernando et al. (18) reported that, at pH 7.5, rabbit skeletal muscle FDPase was inhibited by FDP concentrations greater than 2.5×10^{-6} M. However, in the presence of Mn^{2+} and at alkaline pH, no inhibition of the enzyme was observed at the highest FDP concentration used.

The presence of Mn^{2+} or Mg^{2+} was found to be required for activity in the FDPases of rat liver (8), rabbit liver (69), and rabbit skeletal muscle (18). Bonsignore et al. (8) found that, for rat liver FDPase, optimal concentrations were 1×10^{-4} M for Mn^{2+} and 7×10^{-4} M for Mg^{2+}. Bonsignore added that the results were not dependent on the nature of the anion utilized. Pontremoli et al. (69) reported that rabbit liver FDPase in the presence of either Mn^{2+} or Mg^{2+} exhibited little activity in the neutral pH range but a maximum activity at pH 9.0. McGilvery (43) reported that for rabbit liver FDPase, in the presence of Mg^{2+}, the pH optimum is influenced by the concentration of the cation. He found that increasing Mg^{2+} concentrations shifted the pH optimum of liver FDPase from pH 9.0 to pH 6.0. In the case of skeletal muscle FDPase, Fernando et al. (18) found that, with 5-10 mM Mg^{2+}, the rate of hydrolysis of FDP by the enzyme showed a maximum at neutral pH. When the concentration of Mg^{2+} was decreased to 0.5 mM, the pH of maximum activity shifted to 8.0. In the case of Mn^{2+}, 0.5 mM Mn^{2+} gave highest activity at pH 9.3, with a second, smaller maximum at pH 7.0. However, the liver and muscle FDPases of rabbit appeared similar in that increasing Mg^{2+} concentrations shifted the pH optima towards the neutral region while Mn^{2+} gave a predominantly alkaline pH optimum. The cation requirements for pig or rabbit kidney FDPase has not been

reported. Various researchers (17, 37, 45), however, have conducted assays for these enzymes in the presence of 7.5 mM Mg^{2+}, 12 mM Mg^{2+}, or 0.5 mM Mg^{2+}.

The final factor in the consideration of the catalytic properties of the various FDPases is the effect of pH on their respective activities. Bonsignore et al. (8), reported that, for rat liver FDPase, the maximal rate of cleavage of FDP occurred at about pH 9.0. Pontremoli et al. (71) also reported a pH optimum of about pH 9.0 for rabbit liver FDPase in the presence of either Mn^{2+} or Mg^{2+}. However, McGilvery (43) reported the pH optimum of rabbit liver FDPase was dependent upon the Mg^{2+} concentration. Fernando et al. (18) reported that the pH optimum for FDP hydrolysis by rabbit muscle FDPase was dependent on both the nature of the activating cation and its concentration. These aspects have been discussed in the preceding paragraph. No information was available on the pH optimums of any of the purified mammalian kidney FDPases (20, 21, 22). The pH for the assay of pig kidney and rabbit kidney FDPases were 8.2 in the presence of 0.5 mM EDTA (37), 8.0 in the presence of 5 mM cysteine (45), and 7.5 with no added chelating agents (17).

The basis for the inclusion of 0.5 mM EDTA in the pig kidney FDPase reaction mixture (37) was that the FDPases purified from several sources (5, 73, 75) were found to be activated within the physiological pH range

by the presence of EDTA. Pontremoli et al. (69, 71) did not report whether EDTA was required for rabbit liver FDPase activity in the physiological pH range. However, in several of their subsequent reports on rabbit liver FDPase (59, 60, 63, 70), low concentrations of EDTA were present in assays conducted in the physiological pH range. Recently, Nakashima et al. (50) reported that although a 2-fold increase in the activity of liver and kidney FDPases at pH 7.5 in the presence of very low concentrations (5 mM) of EDTA was observed, the pH optimum was still around pH 9.0 in the presence of either Mn^{2+} or Mg^{2+}. They suggested that FDPases require a chelating agent for maximal activity, the best, to date, being EDTA. Fernando et al. (18) reported that rabbit muscle FDPase exhibited no requirement for EDTA. They also stated that no effect of EDTA was observed when the enzyme was assayed with Mg^{2+} or Mn^{2+} at neutral or alkaline pH or preincubated with EDTA (1.0 mM).

Several researchers (17, 22, 73, 75) have also observed that cysteine stimulated the activity of FDPases from various sources. Bonsignore et al. (8) and Fernando et al. (18), in contrast, reported that rat liver and rabbit muscle FDPases did not have a requirement for cysteine.

As all glycolytic reactions take place around pH 7 (36), it appears that rabbit liver and rabbit muscle FDPases, in order to be maximally active at this pH,

must be activated by some physiological compound. Pontremoli *et al.* (69) reported that the incubation of rabbit liver FDPase with 2,4-dinitrofluorobenzene, in the presence of Mn^{2+}, gave a 3-to 4-fold increase in activity at pH 7.5 and a small decrease in activity above pH 9.0. In the presence of Mg^{2+}, there was a decrease in activity over the entire pH range, but, again, the activity at pH 7.5 tended to approach that at pH 9.1. In a subsequent report, Pontremoli *et al.* (68) stated that dinitrophenylation of a single cysteine residue caused the activation of rabbit liver FDPase. They suggested that dinitrophenylation or increasing the pH caused dissociation of this single sulfhydryl group and, hence, the activity of the enzyme was enhanced. They also suggested that, as dinitrophenylation of the enzyme did not block the enzyme activity, this sulfhydryl group can not be part of the active site itself but must be located at an allosteric site, such that ionization or dinitrophenylation resulted in a change in conformation of the protein.

In the case of rabbit muscle FDPase, Fernando *et al.* (20) reported that dinitrophenylation of the enzyme, in the presence of Mn^{2+}, gave activation over the entire pH range. The activation was greatest at pH 7.5, at which pH the activity was equal to that at pH 9.2. When Mg^{2+} was used as the cation, dinitrophenylation of the enzyme caused an overall decrease in activity.

By analogy with the rabbit liver FDPase, they assumed that dinitrofluorobenzene was reacting with cysteine residues.

In another report, Pontremoli et al. (70) stated that, in the presence of Mn^{2+} and at pH 7.5, rabbit liver FDPase was rapidly activated 4-fold upon incubation with cystamine. This activation appeared to be due to a sulfhydryl-disulfide exchange reaction between protein sulfhydryl groups and cystamine, which was reversible by treating the modified protein with a sulfhydryl reagent such as glutathione or cysteine. Pontremoli et al. suggested that this reaction may play a physiological role in the regulation of FDPase activity. Support is given to this idea by reports of cystamine occurring in mammalian liver as a product of pantothenic acid metabolism (15, 21). On the other hand, Fernando et al. (20) reported that rabbit muscle FDPase is not activated by disulfide exchange with cystamine, which has not been reported to occur in muscle.

Recently, Nakashima et al. (51) reported that, at pH 8.5, rabbit liver FDPase is activated 4-to 5-fold with oxidized coenzyme A (CoA) or acyl carrier protein via a disulfide exchange reaction. They found, however, that the enzyme could be activated 4-fold at neutral pH by reduced CoA in what appeared to be an oxidation reaction. They reported that this activation

could be completely reversed by treatment with sulfhydryl reagents, such as reduced glutathione or cysteine. They suggested that modulation of FDPase activity by modification of sulfhydryl groups, as reported for dinitrofluorobenzene (67, 68, 69), may be carried out by two natural compounds, CoA and acyl carrier protein. They also suggested that the activated form of the enzyme appeared to be a derivative in which the activator is linked to the protein by a disulfide bridge. In a subsequent report, Nakashima et al. (50) reported similar although smaller activation of rabbit kidney FDPase by CoA and acyl carrier protein. Activation of the kidney enzyme was observed at pH 7.0 with reduced CoA or at pH 8.5 with oxidized CoA.

Recently, Pontremoli and Horecker (65) cited unpublished data indicating 10-fold activation of rabbit liver FDPase at pH 7.5 by homocysteine in the presence of either Mn^{2+} or Mg^{2+}. They reported that the reaction is rapid and is even more rapidly reversible by glutathione, cysteine or other sulfhydryl compounds.

On the basis of the above discussion, it may be concluded that liver and muscle FDPases are different in that the muscle enzyme does not require EDTA for maximal activity at physiological pH whereas the liver enzyme does. They are similar in many other respects.

Both exhibit maximal activities at pH 9 in the presence of low concentrations of Mn^{2+} or Mg^{2+}. With high Mg^{2+} concentration, and the presence of EDTA in the case of the liver, maximum activity is observed in both enzymes at neutral pH. Mammalian liver and muscle FDPases also appear similar in their capacity to be activated, in the presence of Mn^{2+}, at pH 7.5 by reaction with dinitrofluorobenzene. They also appear similar in that dinitrophenylation of a single sulfhydryl group causes the activation of the enzyme. However, the mammalian liver and muscle FDPases seem different in that the liver enzyme was activated in the physiological pH range by cystamine while muscle FDPase was not activated. It is noteworthy that rabbit liver and rabbit kidney FDPases have been reported to be activated in the physiological pH range by several physiological compounds such as cystamine, CoA, acyl carrier protein, and homocysteine. It is also noteworthy that this activation is reversed by physiological sulfhydryl reagents, such as glutathione and cysteine.

A Comparison of the Allosteric Properties

FDPase is considered to be an allosteric enzyme. The basis for this statement is its exhibition of several characteristics of allosteric enzymes, such as, its inhibition by adenosine 5′-monophosphate (AMP) (88) and its regulatory function in gluconeogenesis (92). Taketa and Pogell (88), Newsholme (52), and Mendicino

and Vasarhely (46) were among the first investigators to report the inhibition of FDPase activity by AMP. Since then, sensitivity to the presence of AMP has been reported as a property of FDPases from widely different biological sources, (6, 7, 18, 22, 37, 38, 46, 60, 74, 75, 77, 80).

Specific interest, in this review, lies in the inhibition of mammalian FDPases by AMP. Pontremoli et al. (60) reported that 1×10^{-4} M AMP caused a 64% inhibition of rabbit liver FDPase in the presence of Mg^{2+} or 43% inhibition in the presence of Mn^{2+}. Marcus (38) reported that, at pH 7.5, the presence of 1.1×10^{-4} M AMP inhibited pig kidney FDPase by 78%. Fernando et al. (18) reported that, at neutral pH, the presence of 5×10^{-7} M AMP inhibited rabbit muscle FDPase by more than 90%. Similar sensitivities of skeletal muscle FDPases from a variety of animals have been previously reported (30, 55, 77). Thus, it seems that mammalian muscle FDPase is more sensitive to AMP inhibition than mammalian liver or kidney FDPases.

A Comparison of Molecular Properties

From the preceding discussion, there appears to be several differences in the chemical properties among the FDPases isolated from different mammalian tissues. A comparison of the molecular properties of the various tissue FDPases also shows that certain properties of the enzymes are different while others are similar.

The properties to be compared are electrophoretic mobility, immunological inactivation, amino acid analysis, molecular weight, and subunit structure.

In a comparison of the electrophoretic mobilities of rabbit tissue FDPases, Enser et al. (17) reported that, in pH 6.5 buffer, liver and kidney FDPase migrated towards the negative electrode with similar mobilities. They added that a mixture of these two enzymes migrated as a single band. In contrast, the muscle FDPase had little mobility under the same conditions. This is supported by Fernando et al. (20) who reported that, at pH 7.3, the electrophoretic mobilities of rabbit muscle and liver FDPases were different. Rabbit muscle FDPase migrated towards the anode while the liver enzyme remained at the origin. When a mixture of the two enzymes was analyzed, the two bands were clearly separated.

Enser et al. (17) used immunological techniques in order to detect any possible structural relationships between the rabbit FDPase isozymes. Ouchterlony double diffusion analysis on agar and inhibition of FDPase activity with anti-serum to rabbit liver FDPase showed no immunological differences between rabbit liver and kidney FDPases. However, the anti-serum gave little response to rabbit muscle FDPase in either method used. These observations provide additional evidence for two isozymic forms of the enzyme,

one present in muscle and another in kidney and liver tissue.

A comparison of the isozymes of a given protein on the basis of amino acid composition is a good indication of the degree of similarity that exists between the isozymes. Fernando et al. (18) reported that rabbit muscle and liver FDPase differed significantly in amino acid composition. They found that muscle FDPase contained more glutamic acid, leucine, tyrosine, and arginine, whereas the liver enzyme was richer in aspartic acid, isoleucine, phenylalanine, lysine, and methionine. Krulwich et al. (31) reported that rabbit liver and kidney FDPases vary significantly in their content of some amino acids. They found that rabbit liver FDPase contained more lysine and aspartic acid whereas the kidney enzyme contained more arginine and proline.

The molecular weights of the isozymes of a given protein are generally the same (28, 39, 40, 49). The FDPases from mammalian liver, kidney, and skeletal muscle sources have a molecular weight of approximately 130,000 g per mole. Pontremoli et al. (71) reported that sedimentation analysis in a sucrose density gradient yielded an $S_{20,w}$ value of 7.2 for rabbit liver FDPase. Assuming a spherical protein with a partial specific volume of 0.725 cm^3 per g, they estimated the molecular weight to be 130,000 g per mole.

Pontremoli et al. (67) estimated the molecular weight of the same enzyme by sedimentation equilibrium methods and, from a partial specific volume of 0.74 cm^3 per g estimated from the amino acid composition, estimated the molecular weight to be 127,000 ± 13,000 g per mole. Mendicino et al. (45) reported that the molecular weight of pig kidney FDPase, estimated by sucrose density gradient centrifugation, was about 130,000 with an $S_{20,w}$ of 7.4 and a partial specific volume of 0.735 cm^3 per g. Sedimentation velocity experiments with the same enzyme yielded an $S_{20,w}$ value of 7.5. The partial specific volume of 0.735 cm^3 per g was calculated from the amino acid composition, and a diffusion constant of 5.44×10^{-7} cm^2 sec^{-1} was calculated from Sephadex G-200 chromatography data. The $S_{20,w}$, partial specific volume, and diffusion constant were combined using the procedure of Svedberg (86) to yield an estimated molecular weight of 129,500 ± 700 g per mole. The molecular weight of pig kidney FDPase was also determined by chromatography on Sephadex G-200, which yielded a value of about 130,000 g per mole. Krulwich et al. (31) estimated the molecular weight of rabbit kidney FDPase to be about 139,500 g per mole, as estimated by sedimentation equilibrium. Fernando et al. (18), by means of sucrose density gradient centrifugation, estimated the molecular weight of rabbit skeletal muscle FDPase to be 133,000 g per mole, using an $S_{20,w}$ value

of 7.04 and an assumed partial specific volume of 0.725 cm^3 per g.

Pontremoli *et al.* (66) reported that rabbit liver FDPase was dissociated into two subunits by exposure to pH 2.0. They also stated that analysis of the carboxy- and amino-terminal amino acids confirms the existence of two polypeptide chains and suggested that these were not identical. Sia *et al.* (82) reported that rabbit liver FDPase was dissociated in the sodium dodecyl sulfate, or by treatment with malic anhydride, and yielded a mixture of two distinct polypeptides, with molecular weights corresponding to approximately 29,000 and 36,000 g per mole respectively. They also stated that the presence of two different peptide chains was supported by hydrazinolysis experiments which yielded nearly two equivalents each of alanine and glycine. They suggested that, since FDPase has been shown to bind four equivalents each of substrate and AMP, the species detected may be dimers of catalytic and regulatory subunits, respectively. Krulwich *et al.* (31) stated that rabbit kidney FDPase, upon treatment with malic anhydride, dissociated into two peptide species which corresponded to molecular weights of 32,800 and 39,620 g per mole, as determined by sedimentation equilibrium experiments. No information was available, from the literature, on the subunit structure of mammalian muscle FDPase.

C. Physiological Roles of the Fructose 1,6-Diphosphatase Isozymes of Mammalian Tissues

An important consideration in a discussion of an enzyme is the physiological role of the enzyme. FDPase is considered to be a gluconeogenic enzyme involved in the synthesis of glucose or glycogen (92). However, in the case of the FDPases from mammalian tissues, there may be a difference in physiological roles in that, while liver and kidney are gluconeogenic organs (9, 65), there is no net synthesis of glucose or glycogen from pyruvate in mammalian muscle (30). Consequently, the elucidation of the role of FDPase in the mammalian liver, kidney, and muscle tissues may help to explain some of the differences in carbohydrate metabolism found between these tissues.

As mammalian liver and kidney are both gluconeogenic organs (9, 92), their constitutive FDPases are probably primarily active in the synthesis of carbohydrate from lactic acid, as well as from the breakdown products of protein and lipid. Pontremoli and Horecker (65), in a review of the properties of rabbit liver FDPase, suggested that liver FDPase may be regulated by inhibition by AMP and induction of activity at neutral pH by compounds which modify specific sulfhydryl groups in the protein. They report evidence that there was no significant change in AMP concentration under conditions which altered the rate of glycolysis and gluconeogenesis

and that the conversion of FDP to fructose 6-phosphate was a crossover point between gluconeogenesis and glycolysis. On this basis, they suggested a scheme for the transition from glycolysis to gluconeogenesis in liver, based on the changes in the concentration of FDP. They suggested that FDP, at high concentrations, promoted glycolysis and inhibited gluconeogenesis; the latter by virtue of the fact that FDP greatly enhanced the affinity of FDPase for the allosteric inhibitor AMP (62, 78, 91). Glycolysis would then be enhanced. On the other hand, when the level of FDP fell, due to inhibition of phosphofructokinase (EC 2.7.1.11) by the breakdown products of lipids and proteins, the inhibition of FDPase by AMP was removed. Gluconeogenesis would then be enhanced. Pontremoli and Horecker (65) suggested a mechanism for control of FDPase activity based on the rate of hydrolysis of FDP exceeding the rate at which fructose 6-phosphate was converted to glucose and glycogen. Hexose monophosphate then accumulated and was presumably passed through the pentose phosphate pathway, resulting in increased NADPH production. Conceivably the excess NADPH then caused the reduction of glutathione to the reduced form of glutathione. This, in turn, caused the inactivation of FDPase. In avian hepatic tissue, this type of control may not be involved as the activities of enzymes involved in the pentose phosphate pathway are

very low (24).

In mammalian muscle tissue, it was found that resynthesis of glycogen from lactate cannot be a major metabolic process in muscle tissue (30). Glucose or glycogen can be synthesized from lactate only with the presence of the key, rate-limiting enzymes of gluconeogenesis, namely, FDPase, pyruvate carboxylase and phosphopyruvate carboxylase. The virtual absence of the pyruvate carboxylase in muscle tissues precluded the synthesis of phosphoenolpyruvate from pyruvate, and hence, gluconeogenesis. Consequently, the finding of significant amounts of FDPase activity in cat skeletal muscle homogenates (30) led to the problem of determining the physiological role of FDPase in mammalian muscle tissue.

Krebs and Woodford (30) postulated that, in muscle tissue, α-glycerophosphate was a key intermediate in "α-glycerophosphate —— pyruvate glycolysis". The need for this cycle arose because, under aerobic conditions, pyruvate diffused into the mitochondria and underwent rapid oxidation. This decreased the amount of pyruvate available for lactate formation and, as a result, cytoplasmic levels of NAD decreased. This allowed for inhibition of the glyceraldehyde 3-phosphate dehydrogenase system and, therefore, glycolysis. Under conditions of low pyruvate concentration, reduction of dihydroxyacetone phosphate to α-glycerophosphate served

as an alternative mechanism for oxidizing $NADH_2$ to NAD^+ in the cytoplasm. Thus, the "α-glycerophosphate —— pyruvate glycolysis" allowed the breakdown of glucose to proceed and maintained a supply of oxidizing material to the mitochondria when the pyruvate concentration, owing to the rapid oxidation of pyruvate, was inadequate. Unlike lactate, however, α-glycerophosphate could not diffuse out of muscle cells. Therefore, it was oxidized via glycolysis or reconverted into carbohydrate within the muscle. This reconversion was thought to be the raison d'etre for the presence of FDPase in muscle tissue.

Opie and Newsholme (54) expanded on the above theory (30). They suggested that, in order to maintain rapid glycolysis, the requirement for oxidation of the NAD^+ that was produced extramitochondrially was met not only by the α-glycerophosphate —— dihydroxyacetone phosphate cycle but also by the malate —— oxaloacetate cycle. They also provided evidence which supported the idea that oxidation of $NADH_2$ by these cycles may be more important in muscle tissues that depended upon glycolysis for most of their energy (that is, white muscle rather than red muscle). Their theory of glycogen synthesis from α-glycerophosphate and malate was supported by the coincident presence of FDPase and phosphoenolpyruvate carboxykinase activities, along with the activities of the enzymes of these cycles. It was

suggested that the activities of these cycles were controlled by the concentrations of ∝ -glycerophosphate and malate, so that the function of FDPase and phosphoenolpyruvate carboxykinase in white muscle might be to decrease the concentrations of accumulated intermediates of the two cycles, thereby inhibiting the activities of these cycles upon the cessation of muscular activity. Thus, Opie and Newsholme suggested that the role of FDPase in skeletal muscle was to control the ∝ -glycerophosphate cycle and, thereby, to control, to a degree, the rate of glycolysis.

Recently, a second hypothesis on the role of FDPase in mammalian muscle has been proposed by Newsholme and Crabtree(53). They suggested that FDPase was present in skeletal muscle to provide cycling of fructose 6-phosphate and FDP, when the muscle was at rest, by the simultaneous activity of FDPase and phosphofructokinase. This continuous cycle would increase the sensitivity of phosphofructokinase to activation by AMP by providing a threshold response of the enzyme to changes in the AMP concentration. That is, a four-fold increase in phosphofructokinase activity would only require a three-fold increase in AMP concentration. They further stated that increased AMP concentration would inhibit the FDPase activity and ensure that, when energy was required for muscular contraction, loss of energy and restriction of the rate

of glycolysis at the stage of fructose 6-phosphate phosphorylation was reduced to a minimum.

Materials and Methods

A. Materials

Chicken livers were purchased from Dunn-Rite Food Products Limited, Winnipeg. Chicken breast muscle was obtained from growing hens taken from the University Poultry flock.

Phosphocellulose resin, tris (hydroxymethyl) aminomethane (Tris), fructose 1,6-diphosphate, Sigma grade and grade II, Ponceau S, nicotinamide adenine dinucleotide phosphate (NADP), phenazine methosulfate, nitro blue tetrazolium chloride, 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), bovine serum albumin, and ovalbumin were purchased from Sigma. Ammonium sulfate, (Ethylene dinitrilo) tetraacetic acid (EDTA), 2-mercaptoethanol, sodium malonate, magnesium chloride, potassium phosphate, sodium chloride, trypan blue, sucrose, sodium hydroxide, urea, and potassium chloride were purchased from J. T. Baker. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49), phosphohexose isomerase (EC 5.3.1.9), and rabbit muscle aldolase (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. Calbiochem standard amino acid mixture and glycylglycine were purchased from Calbiochem; DEAE (DE-52)-cellulose resin was from Whatman; guanidine

hydrochloride was from Mann Research Laboratories, and sulfuric acid was purchased from Fisher Scientific Company. Standard amino acid mixture was purchased from Beckman; norleucine was from Nutritional Biochemical Company; and carrier ampholyte was from LKB Produkter.

B. General Methods

Absorbance measurements were made with either a Unicam SP-- 800 or a Gilford 2400 automatic recording spectrophotometer.

Protein concentrations in the early purification steps were measured by the method of Lowry et al. (35) using bovine serum albumin as a reference protein (milligrams of liver FDPase per milliliter equals milligrams bovine serum albumin standard per milliliter x 0.86 while milligrams of breast muscle FDPase per milliliter equals milligrams bovine serum albumin standard per milliliter x 0.78). For pure enzyme preparations, the absorbance at 280 mμ was also used (milligrams of either liver or breast muscle FDPase per milliliter equals 1.36 x absorbance at 280 mμ for a 1 cm light path). The above conversions were standardized against the amino acid content of each enzyme as described under amino acid analysis.

Since Tris has a high temperature coefficient for pH, the temperature at which the pH was measured is given in parentheses in those cases where temperature

is not obvious.

Enzyme Assays

Unless otherwise stated, all assays were run at 30°C, the rate of change of NADP concentration was measured at 340 mμ.

For the routine assay of FDPase activity, the rate of formation of fructose 6-phosphate was measured spectrophotometrically by following the reduction of NADP in the presence of excess phosphohexose 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 phosphohexose isomerase-0.0025 to 0.010 units of enzyme-0.15 mM FDP. The reaction was initiated with the addition of FDP or enzyme.

The aldolase (EC 4.1.2.13), triose phosphate isomerase (EC 5.3.1.1), glycerophosphate dehydrogenase (EC 1.1.1.8), and glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) assays were the same as those described by Marquardt (39), Marquardt and Brosemer (41), and Marquardt et al. (42).

The lactate dehydrogenase (EC 1.1.1.27), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), malic enzyme (EC 1.1.1.40), and pyruvate kinase (EC 2.7.1.40), assays were those of Kornberg (29), Langdon (33), Wise and Ball (94), and Bailey et al. (4), respectively.

The phosphohexose isomerase (EC 5.3.1.9) assay was the same as the FDPase assay except for auxillary enzyme. Excess FDPase was substituted for phosphohexose isomerase.

In all cases, a unit of enzyme activity is defined as that amount of enzyme catalyzing the disappearance of 1 μmole substrate per minute under standard conditions.

Zone Electrophoresis

Cellulose acetate electrophoresis was performed at 5°C on 17 x 2.5 cm Sepraphore III strips in a Gelman electrophoresis chamber. The buffer systems used are described in the text. The strips were stained for protein with Ponceau S. The FDPase activity stain was a modification of that reported by Penhoet et al. (56) for aldolase. A 0.6% Noble agar solution (w/v) in 50 mM Tris-1 mM EDTA (pH 7.8 at 30 C) containing 0.5 mM FDP-25 mM $MgCl_2$ -0.5 mM NADP-0.04 mg/ml phenazine methosulfate-0.4 mg/ml mitro blue tetrazolium chloride-2.5 μg/ml phosphohexose isomerase-2.5 μg/ml glucose 6-phosphate dehydrogenase was poured into shallow trays (4 ml/tray) at 42°C and allowed to solidify at 4°C. After electrophoresis, the cellulose acetate strips were placed on this agar and incubated at 37°C for 10 to 20 minutes to allow color development.

Immunological Analysis of Fructose 1,6-Diphosphatase with Specific Anti-serum

Anti-serum to chicken liver FDPase was prepared in the rabbit by subcutaneous injections of the chicken

liver enzyme emulsified in Freund's complete adjuvant. The injection schedule involved five weekly injections (1 to 2 mg protein per injection) of recrystallized enzyme. Immune serum (anti-liver FDPase) was obtained from clotted blood drawn weekly on the fifth to eleventh week after the first injection and was stored at -5°C until used.

Immunological analysis of FDPase with specific anti-serum was carried out using a modified two-dimensional gel diffusion (Ouchterlony) method as described by Campbell et al. (10). The agar plates contained 0.8% agar (w/v) in 2 mM EDTA-50 mM glycylglycine-1.5% NaCl (w/v)-0.01% trypan blue (w/v) (pH 7.5 at 20°C). The reaction was carried out for 4 to 6 days at room temperature.

Liver extracts of rabbit, rat, budgerigar, and turkey, and extracts of liver, kidney, heart muscle, brain, and breast muscle of chicken were prepared in order to estimate the extent of cross-reaction of the anti-liver FDPase with FDPases from various species and tissues. Ten grams of each tissue was homogenized in 10 ml of 0.1 M Tris-50 mM EDTA-10 mM 2-mercapto-ethanol-0.1 M KCl (pH 7.5 at 30°C). In the case of the budgerigar, 0.5 g of liver was homogenized in 1.0 ml of the above buffer. The homogenates were then centrifuged at 200,000 x g for 20 min and the super-natants were then assayed for FDPase activity.

Isoelectric Point

The isoelectric points of the avian FDPases were determined by the method of Svensson (87) with a LKB 8101 electrofocusing column and 8% carrier ampholyte "Ampholine" (pH 3-10 or 7-10). The method outlined in the LKB 8100 Ampholine Electrofocusing Equipment Instruction Manual (LKB-Produkter AB S-161 25 Bromma 1, Sweden) was followed. A 1% H_2SO_4 solution (w/v) was added in a 50% sucrose solution (w/v) to the anode end of the column. A 50 to 0% sucrose gradient, containing ampholyte and 3 mM 2-mercaptoethanol, was applied to the column. After one-half of the gradient had been applied, the purified enzyme solution was added in equal portions to each of the gradient-forming mixing chambers. After all of the gradient was applied, a 5% KOH solution (w/v) was added to the cathode end of the column. Electrofocusing was then carried out at 10°C by applying a maximum power of 3 watts. After each focalization run, 2 ml fractions were collected from the bottom of the column. The enzyme activity, absorbancy at 280 mμ, and pH values of each fraction were determined.

Liver FDPase (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 initially focalized for 90 h using pH 3-10 "Amphroline". After the analyses of the various fractions were carried out, those fractions

containing most of the FDPase activity were pooled and added to the electrofocusing column. In this case, an ampholyte pH range of 7 to 10 was used. Electrofocusing time was 60 h.

The procedure for the determination of the isoelectric point of muscle FDPase was essentially the same as that for liver FDPase. However, the initial protein sample added contained 2.4 mg of protein in 1.2 ml of the above buffer. Both electrofocusing determinations were carried out for approximately 48 h. Also, the initial fractions collected were monitered only for pH and FDPase activity while the second set of fractions were monitered for pH, absorbancy at 280 mμ, and FDPase activity.

Amino Acid Analysis

Amino acid analyses of chicken liver and breast muscle FDPases were performed, on separate occasions, by the method of Moore and Stein (47) with a Beckman Spinco model 116 automatic amino acid analyzer using Calbiochem amino acid mixture, (lot No. 893001) and Beckman amino acid mixture (lot No. CM 103, Nov. 1969) as reference standards. Each standard contained 0.1 μmole /ml norleucine.

In the first analysis, crystalline chicken liver FDPase was dialyzed against 5 mM Tris-0.5 mM EDTA-2 mM 2-mercaptoethanol (pH 7.5 at 10°C) and 0.41 mg aliquots were hydrolyzed at 112 ± 2°C for 24, 48, and

72 h in vaccuum-sealed tubes with 6 M HCl. After hydrolysis, the samples were dried under vaccuum. Cysteine was oxidized to cystine (47) and 0.16 mg of hydrolyzed protein and 0.1 μmole of norleucine were applied to the column. Amino acid analysis was carried out on each hydrolysate.

In the second amino acid analysis of liver FDPase, the procedure was modified so that the dry matter content could be determined directly from the amino acid analysis (90). In this analysis, crystalline chicken liver FDPase was dialyzed against 20 mM phosphate-2 mM EDTA (pH 6.5) and 1.3 mg aliquots of protein and 0.5 umoles norleucine were hydrolyzed and prepared for amino acid analysis as described above. The amount of protein and norleucine applied to each column were 0.26 mg and 0.1 μmole, respectively.

In the case of chicken breast muscle FDPase, the modified procedure was used for the duplicate 24-, 48-, and 72-h sets of amino acid analyses. FDPase for both sets of determinations was dialyzed against 20 mM phosphate-2 mM EDTA (pH 6.5) and 0.80 mg aliquots of enzyme and 0.4 μmoles norleucine were hydrolyzed. Amino acid analyses were carried out as described above; one-quarter of the hydrolysate volume, equivalent to 0.20 mg of protein, and 0.1 μmoles norleucine was applied to each column.

The tryptophan content of the chicken liver and

breast muscle FDPases was determined spectrophotometrically, according to the method of Edelhoch (13). The change in absorbance at 280 and 288 mμ was followed at 25°C in 3-ml cuvettes (2-ml cuvettes in the case of muscle FDPase). The reaction mixture contained 20 mM phosphate-2 mM EDTA (pH 6.5)-6.7 M ultra-pure guanidine hydrochloride. The amount of dialyzed liver FDPase added was between 2.26 and 4.52 mg protein, while the amount of dialyzed muscle FDPase added was between 0.91 and 1.33 mg protein. In both cases, increase in absorbance was proportional to the amount of enzyme added; the dialyzing solution, which was used as a blank, had no absorbance at either 280 or 288 mμ. The tryptophan content of the liver FDPase was also determined by the method of Goodwin and Morton (25).

The number of reactive sulfhydryl groups in both FDPases were determined by reaction with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) according to the procedure of Ellman (16). The change in absorbance at 412 mμ was followed at 25°C in 3-ml cuvettes. The reaction mixture for liver FDPase contained 10 mM Tris-1 mM EDTA (pH 7.5 at 10°C)-8.5 M urea-10 mM Ellman's reagent while that for the muscle FDPase contained 20 mM phosphate-2 mM EDTA (pH 6.5)-8.5 M urea-10 mM Ellman's reagent. The amount of dialyzed chicken liver FDPase added was between 0.19 and 0.38 mg protein while the amount of chicken breast muscle FDPase added was between 0.13

and 0.40 mg protein. In both cases, the increase in absorbance was proportional to the amount of enzyme added; the dialyzing solution, which was used as a blank, had no absorbancy at 412 mμ.

The dry matter contents of the enzymes were estimated by the method of Walsh and Brown (90). The percent recovery of the known amount of norleucine in the hydrolysate provides a direct measure of the percent recovery of the amino acids of the protein solution. The dry weight of the protein can be calculated from the sum of the weights of the individual amino acids (minus one water molecule per peptide bond). A ratio of the amount of dry protein and the known absorbancy at 280 mμ of the sample was then determined. This ratio was then used as a factor for the conversion of absorbancy at 280 mμ to a dry matter basis. A ratio was also determined, by the same means, for the conversion of the amount of protein in the sample measured by the method of Lowry et al. (35) to a dry matter basis.

An analysis of variance of the amino acid compositions was performed as described in Snedecor and Cochran (84).

Molecular Weight Estimation

The molecular weights and the molecular radii of the liver and muscle FDPases were estimated according to the respective procedures of Leach and O'Shea (34) and Ackers (1).

Sephadex G-200 (40 to 120 μ) was allowed to swell one week at room temperature in 50 mM Tris-5 mM EDTA-

100 mM KCl-2 mM 2-mercaptoethanol-0.1 ml/l pentachlorophenol (100 mg/ml EtOH) (pH 7.5 at 10°C). The fines were decanted several times from the suspension and a portion of the remaining gel was poured into a glass column (57.8 x 1.78 cm). The column was equilibrated and eluted with the above buffer. The various proteins were also dissolved and / or dialyzed against this buffer before being applied to the column. Elutions were carried out at 24°C using a flow rate of from 1.5 to 6 ml/h. The void volume was determined with a solution of 0.5% dextran blue. The column was calibrated with various reference proteins. Duplicate 1.0 ml sample volumes containing 2 levels of bovine serum albumin (10 and 20 mg), ovalbumin (10 and 20 mg) and rabbit muscle aldolase (12 and 29 mg) were applied to the column. For the molecular weight determination of liver FDPase, duplicate 1.0 and 2.0 ml aliquots of liver FDPase (8.4 mg/ml) were applied to the column.

For the molecular weight determination of chicken breast muscle FDPase, the column was calibrated using purified chicken liver FDPase. Duplicate sample volumes applied to the column were 0.5 and 0.8 ml containing about 3.0 mg of protein each. For the muscle FDPase, duplicate sample volumes applied to the column were 0.5 and 1.0 ml containing 3.0 and 6.0 mg of protein respectively.

For both molecular weight determinations, fractions

of 1.5 to 3.0 ml were collected and the absorbance was read at 280 mμ; with the exception of dextran blue, which was measured for absorbance at 600 mμ. Liver and muscle FDPases were also monitered for enzymatic activity. Elution volumes were taken at the concentration maximum, determined by triangulation. The calibration curve was a plot of the log of the molecular weight of a given protein against the ratio of the elution volume of that protein to the void volume (V/Vo) (34). The void volume was corrected for the small amount of column shrinkage that occurred as the experiment progressed.

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-2mM 2-mercaptoethanol, pH 7.5 at 10°C). Sedimentation velocity experiments were carried out with a Spinco model E ultracentrifuge, using schliern optics. Corrections for the effect of temperature on viscosity of water were calculated from data from the Handbook of Chemistry and Physics (42 nd ed.). The viscosities of the buffer relative to water were determined from flow times in an Ostwald viscometer; density measurements were obtained with a pyconometer.

C. Purification of Chicken Liver Fructose 1,6-Diphosphatase

General Comments on Isolation of Liver Fructose 1,6-Diphosphatase

All steps were carried out at 0 to 5°C unless otherwise stated. Ammonium sulfate concentrations were increased by the method of Kunitz (32). For steps with P-cellulose and DEAE-cellulose, the resins were prepared by the procedures described in the Whatman Data Manual and Catalogue, No. 2000 (Mandel Scientific Co. Ltd., 4920 de Maisonneuve Blvd. Suite 10, Westmount, Quebec). The exchangers were equilibrated by washing with 5 x concentrated eluting buffer and eluting buffer (20 mM Tris-2 mM EDTA-5 mM 2-mercaptoethanol, pH 7.5 at 10°C).

Step I. Extraction

Three hundred grams of frozen chicken livers were homogenized, at top speed, for 2 minutes in a Waring blender in 900 ml of 10 mM EDTA-2 mM 2-mercaptoethanol (pH 7.5) at 5°C. The homogenate was centrifuged at 25,000 x g for 30 min and the supernatant was passed through several layers of cheese cloth to remove fat particles.

Step II. Ammonium Sulfate Precipitation

Solid ammonium sulfate was added, with constant stirring, to the extract over a period of 1 h to 47% saturation (250 g/900 ml). After standing 1 h, the extract was centrifuged at 25,000 x g for 20 min and the precipitate was discarded. Solid ammonium sulfate

was then added to the clear supernatant over 1 h to 67% (124 g additional ammonium sulfate/920 ml). After standing overnight, the suspension was centrifuged at 25,000 x g for 20 min. The precipitate was dissolved in approximately 300 ml of eluting buffer and dialyzed for approximately 16 h against two-4 l changes of the same buffer.

Step III. Phosphocellulose Substrate Elution Chromatography

The enzyme from the previous step was centrifuged at 50,000 x g for 15 min and adsorbed onto a phosphocellulose column (39 x 3.7 cm) which had been previously equilibrated against the eluting buffer. The column was washed (10 ml/min) with 1,800 ml of eluting buffer to remove unadsorbed proteins. Similar results were obtained when the column was flushed with 8 l of buffer. FDPase and aldolase were then eluted (5 ml/min) with 600 ml of 2.5 mM FDP in the same buffer. This and the subsequent DEAE-cellulose separation were carried out at room temperature. In order to maintain as low a column temperature as possible, the buffer was cooled, before entering the column, to 0 to 5°C. Eluate temperatures were 10 to 15°C. The collected fractions (50 ml) were immediately placed in an ice bath and the appropriate fractions were pooled. A typical elution profile is given in Figure I.

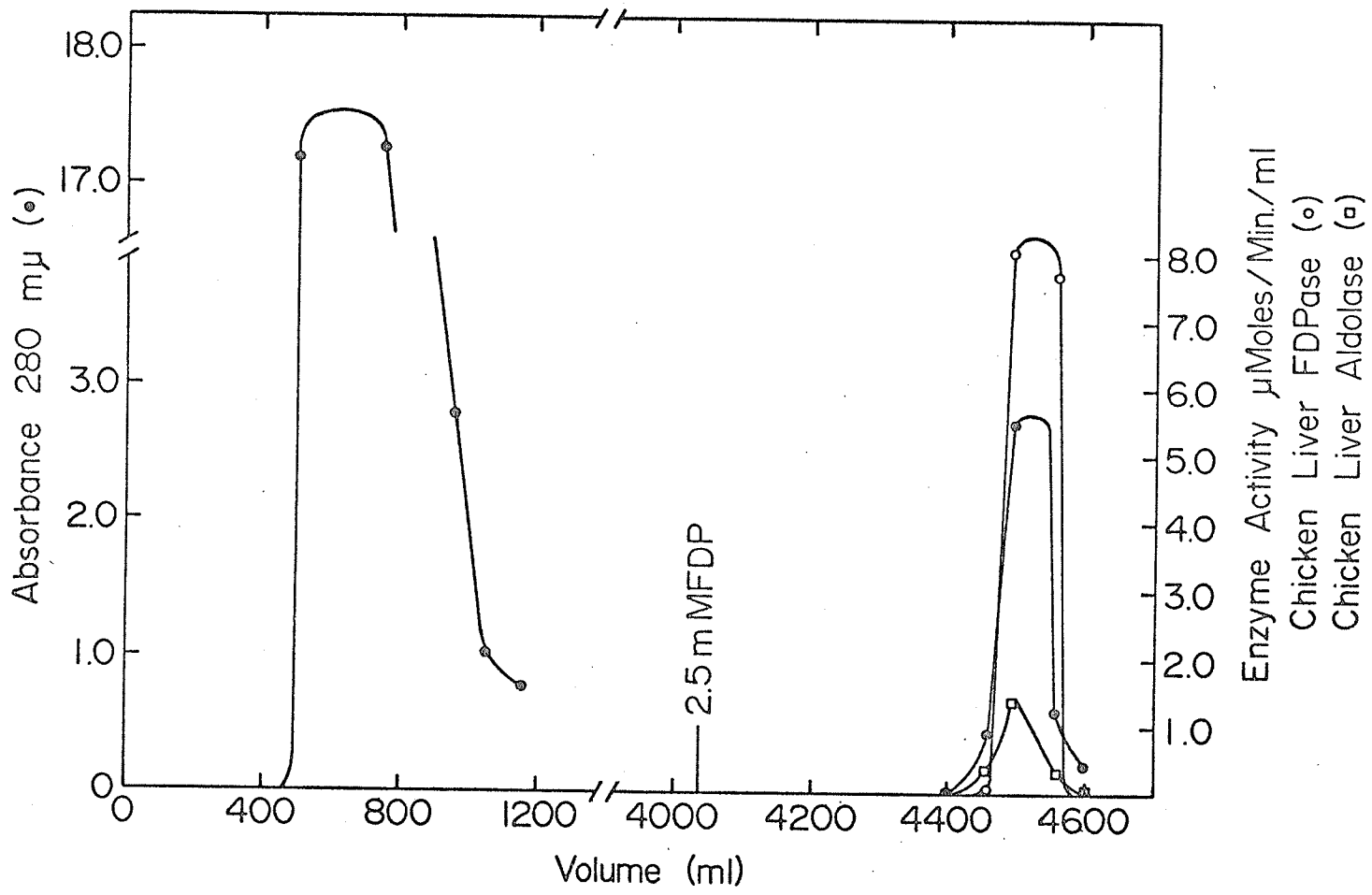


Figure 1

Phosphocellulose chromatography of chicken liver FDPase. For details see text.

Step IV. DEAE-Cellulose (Whatman DE-52) Chromatography

The pooled fractions (110 ml) from the previous step were applied to a DEAE-cellulose column (17 x 3.5 cm) equilibrated with the eluting buffer. The enzyme, which was not bound by the exchanger, was flushed (3 ml/min) from the column with the eluting buffer. Those fractions (42 ml) containing more than 5% of the total activity were pooled and solid ammonium sulfate was added to 80% (71 g/126 ml) to precipitate all of the protein. The precipitated protein was collected by centrifugation (50,000 x g for 20 min), dissolved, and dialyzed against 20 mM malonate-2 mM EDTA-3 mM 2-mercaptoethanol (pH 6.0). After dialysis, the protein concentration was adjusted with buffer to approximately 8 mg/ml. The optimum concentration for crystallisation is from 6 to 12 mg/ml.

Step V and VI. Ammonium Sulfate Fractionation

Solid ammonium sulfate was added over 1 h to 49% (10.4 g/34 ml). A precipitate started to form within a few hours. After standing 6 h, the suspension was centrifuged at 50,000 x g for 20 min. The enzyme was recrystallised using the procedure described above.

D. Purification of Chicken Breast Muscle Fructose 1,6-Diphosphatase

Step I. Extraction

Eight hundred grams of frozen chicken breast muscle were homogenized for 2 min (high speed setting) in a Waring blender 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 x g for 30 min and the supernatant was passed through several layers of cheese cloth.

Step II. Ammonium Sulfate Precipitation

The pH of the extract was adjusted to 8.5 at 10°C with 1 N NaOH, which was mixed in with rapid, thorough stirring. Solid ammonium sulfate was added, with constant stirring, to the extract over a period of 30 min to 65% concentration (802 g/1866 ml). After standing 1 h, the preparation was centrifuged at 25,000 x g for 20 min and the precipitate was discarded. Solid ammonium sulfate was then added to the supernatant, over a period of 30 min, to 80% (214 g additional ammonium sulfate/2000 ml). After standing overnight, the suspension was centrifuged at 25,000 x g for 20 min. The precipitate was dissolved in approximately 100 ml of 50 mM Tris-5 mM EDTA-5 mM 2-mercaptoethanol (pH 8.0 at 10°C) and dialyzed for approximately 16 h against two 4 l changes of the same buffer. This buffer is subsequently referred to as the phosphocellulose eluting buffer.

Step III. Phosphocellulose Salt Gradient Chromatography

The enzyme from the previous step was centrifuged

at 25,000 x g for 10 min and adsorbed onto a phosphocellulose column (26 x 2.8 cm) which had been previously equilibrated against the phosphocellulose eluting buffer. The column was washed (5 ml/min) with 1 l of phosphocellulose eluting buffer to remove unadsorbed proteins. A linear salt gradient (2.5 ml/min) was then applied to the column. This was provided by two reservoirs in series; the first contained 500 ml of phosphocellulose eluting buffer while the second contained an equal amount of the same buffer in 0.7 M KCl. The eluate was collected in fractions which were monitered for aldolase and FDPase enzymatic activities and for absorbancy at 280 mμ. A typical elution profile is given in Figure 2. Those fractions containing FDPase activity were pooled, diluted 1 : 3 with eluting buffer, and adsorbed again onto the phosphocellulose column, which had been rinsed of remaining protein with 500 ml of phosphocellulose eluting buffer containing 1 M KCl and then re-equilibrated with 1 l of phosphocellulose eluting buffer. The column was washed (3 ml/min) with 500 ml of phosphocellulose eluting buffer to remove unadsorbed protein. A linear salt gradient (2 ml/min), similar to the one described above, was then applied to the column. The eluate was collected in fractions which were monitered for FDPase activity and for absorbancy at 280 mμ. A typical elution profile is given in Figure 3. Those fractions containing more than 5% of the total

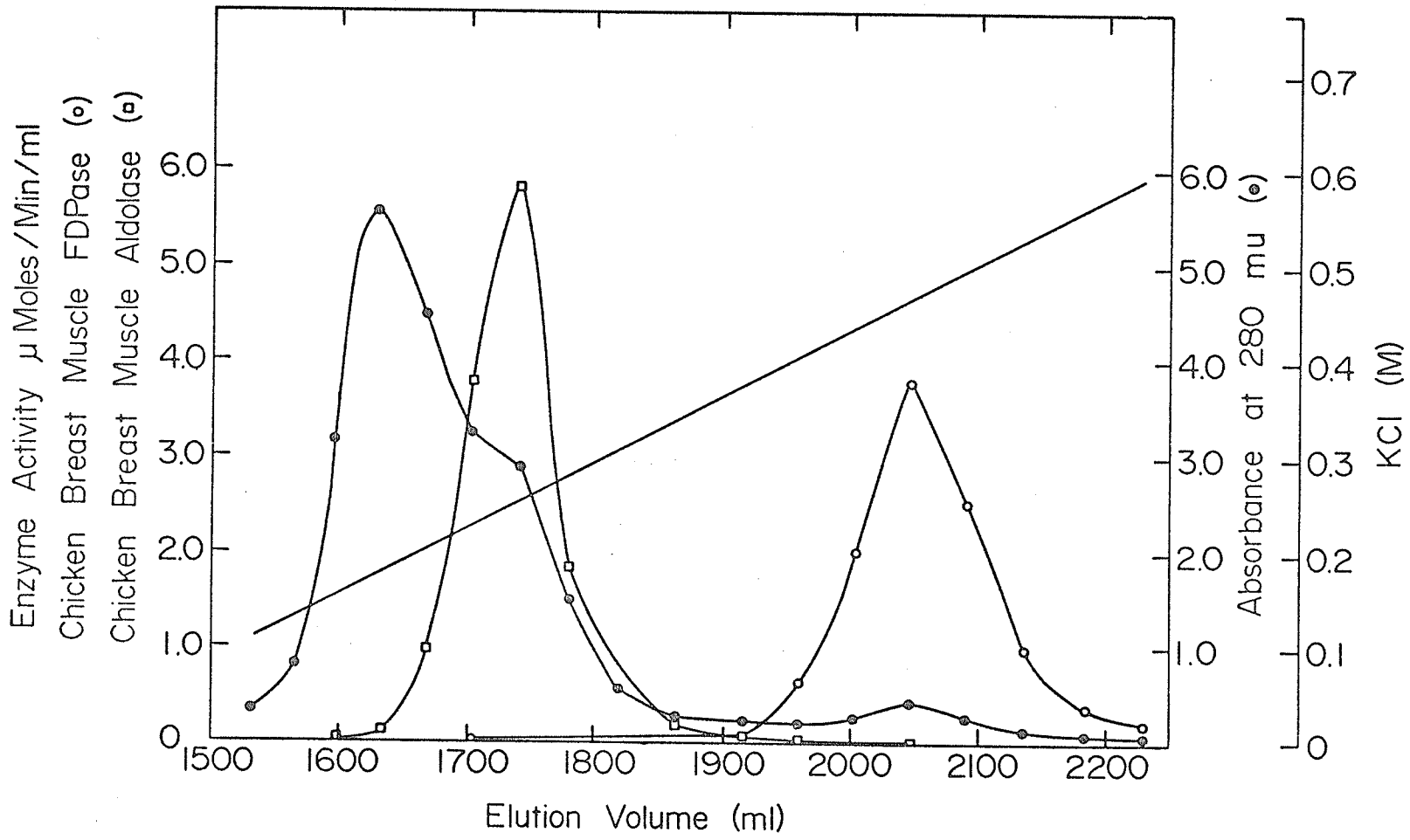


Figure 2

Phosphocellulose chromatography of chicken breast muscle FDPase, initial elution profile. See text for details.a

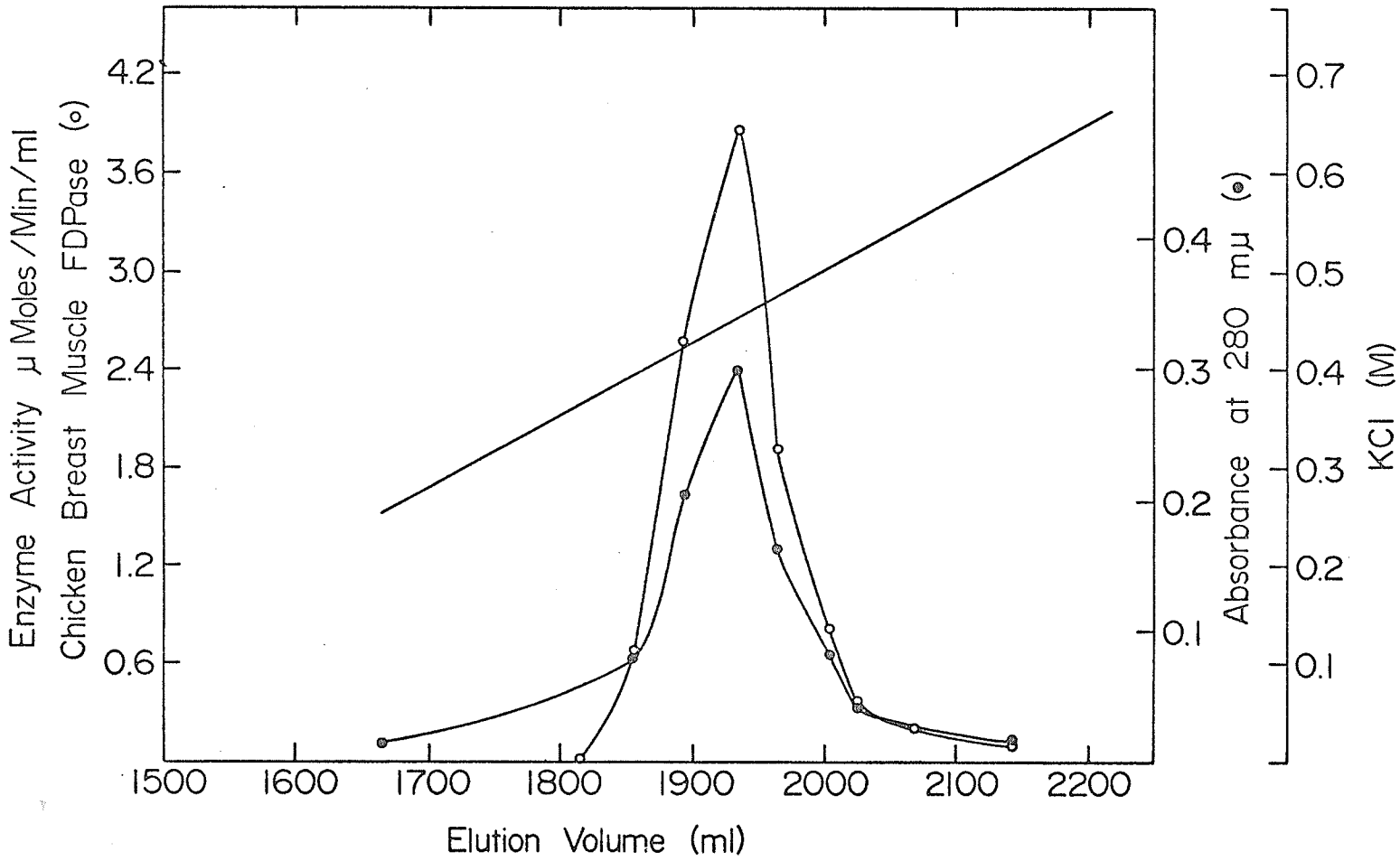


Figure 3

Phosphocellulose chromatography of chicken breast muscle FDPase, second elution profile. See text for details.

FDPase activity were pooled and solid ammonium sulfate was added to 85% (159 g/260 ml) to precipitate all of the protein. The precipitated protein was collected by centrifugation (50,000 x g for 20 min), dissolved, and dialyzed against 50 mM Tris-5 mM EDTA-100 mM KCl-2 mM 2-mercaptoethanol-0.1 ml/l pentachlorophenol (100 mg/10 ml EtOH) (pH 7.5 at 10°C).

Step IV. Sephadex G-200 Chromatography

Sephadex G-200 (40-120 mμ) was prepared as described in the section on molecular weight estimation. A portion of chicken breast muscle FDPase (6.95 mg/2.2 ml) was taken from the protein prepared in Step III and applied to the column previously equilibrated with the buffer in which the enzyme had been dialyzed. The fractions collected were monitered for absorbancy at 280 mμ. Those fractions containing 5% or more of the total eluted protein were pooled and solid ammonium sulfate was added to 85% (10.9 g/18 ml) to precipitate all of the protein. A portion (1.00 ml) of the precipitated protein was taken, centrifuged at 50,000 x g for 15 min to collect the precipitated protein, and dissolved and dialyzed in 300 ml of 50 mM Tris-5 mM EDTA-5 mM 2-mercaptoethanol (pH 8.0 at 10°C).

Results

A. Purification

Purification of Chicken Liver Fructose 1,6-Diphosphatase

A summary of a typical purification procedure of chicken liver FDPase is given in Table I. The overall purification was 60-fold. Twenty percent of the original enzyme activity was recovered.

Cellulose acetate electrophoresis was used to estimate the degree of purity of the various fractions during the purification procedure (Figure 4). A protein stain of the extract indicated the presence, as expected, of many proteins. In contrast, a FDPase enzyme activity stain showed only one, nonmigrating band. As shown in Table I, phosphocellulose chromatography gave a 13-fold purification of the enzyme. However, cellulose acetate electrophoresis indicated the presence of two protein bands in the eluate. The profile of the phosphocellulose chromatography (Fig. 1) indicated that the coincident occurrence of two proteins was due to the presence of chicken liver aldolase and FDPase in the fraction. Crystallisation of the liver FDPase gave a final 60-fold purification (Table I) and, as shown by cellulose acetate electrophoresis, removed the migrating protein band (Fig. 4). Protein and enzyme stains of the pure fraction were superimposable, demonstrating that the activity could be attributed to only one protein band.

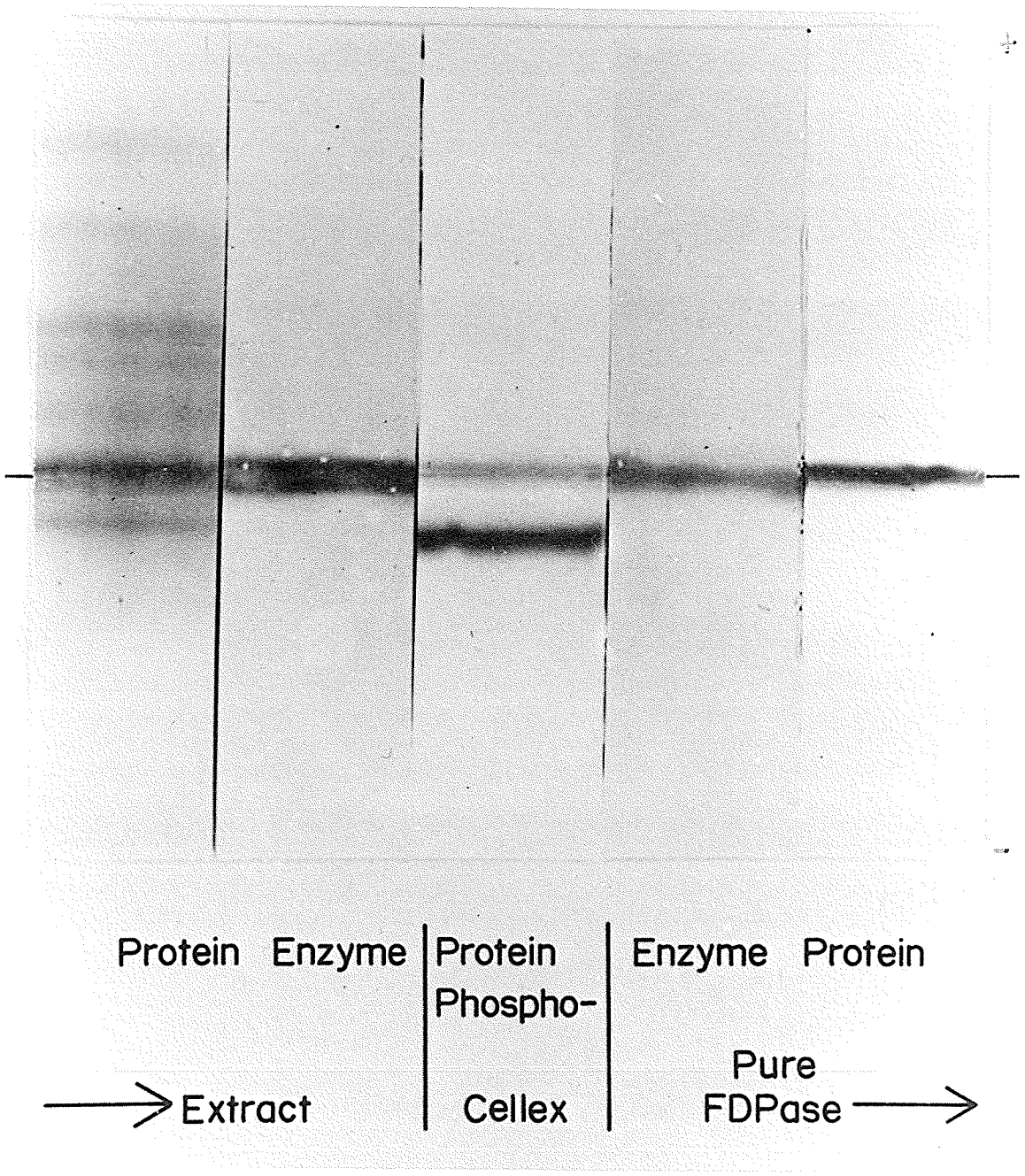
TABLE I

Purification of Chicken Liver FDPase

Fraction		Total enzyme activity	Yield (%)	Specific activity (units / mg protein	Purification (fold)
I	Extract	3249	100	0.23	1.0
II	Ammonium Sulfate Precipitate (47-67%)	1760	54	0.38	1.7
III	Phosphocellulose	928	30	3.13	13.6
IV	DEAE-cellulose concentrated eluate	947	29	4.47	19.4
V	First crystallization	913	28	13.62	59.2
VI	Second crystallization (49% ammonium sulfate)	631	20	13.69	59.5

Figure 4

Electrophoresis of proteins present in several purification fractions (Table I). Approximately 3-6 µl of each fraction was applied to the various strips. Electrophoresis was performed in Gelman barbital-Tris salt mixture (17.8 g/l)-1 mM EDTA-1 mM 2-mercaptoethanol, pH 8.8, at 5°C on 2.5 x 17 cm cellulose acetate strips, (Gelman) at 300 V for 90 min with the origin equidistant from the electrodes. The strips for each fraction were stained for protein with Ponceau S. Strips for the extract and pure enzyme were stained for FDPase activity as described in Materials and Methods.


Protein
Enzyme
Protein
Phospho-
Enzyme
Protein
Extract
Cellex
Pure
FDPase

Purification of Chicken Breast Muscle Fructose 1,6-Diphosphatase

A summary of a typical purification procedure of chicken breast muscle FDPase is given in Table II. The overall purification was 141-fold. Eight percent of the original enzyme activity was recovered.

A FDPase activity stain of the extract showed only one, slightly migrating band (Figure 5). Breast muscle FDPase, purified by sequential double phosphocellulose chromatography (Fig. 2 and 3) and Sephadex G-200 chromatography, exhibited the presence of only one protein band when cellulose acetate electrophoresis was carried out (Fig. 5). Protein and enzyme stains of the pure fraction were superimposable, demonstrating that the activity could be attributed to only one protein band.

B. Criteria of Purity

Criteria of Purity for Chicken Liver Fructose 1,6-Diphosphatase

Chicken liver FDPase was tested for homogeneity by six methods: (a) cellulose acetate electrophoresis (Fig. 4), (b) purification to constant specific activity, (c) absence of other enzymatic activities, (d) immunological analysis, (e) ultracentrifugal analysis, and (f) crystallisation of the enzyme.

The enzyme retained constant specific activity following crystallisation and re-crystallisation (Table I). The final specific activity of 13.7 units/mg protein

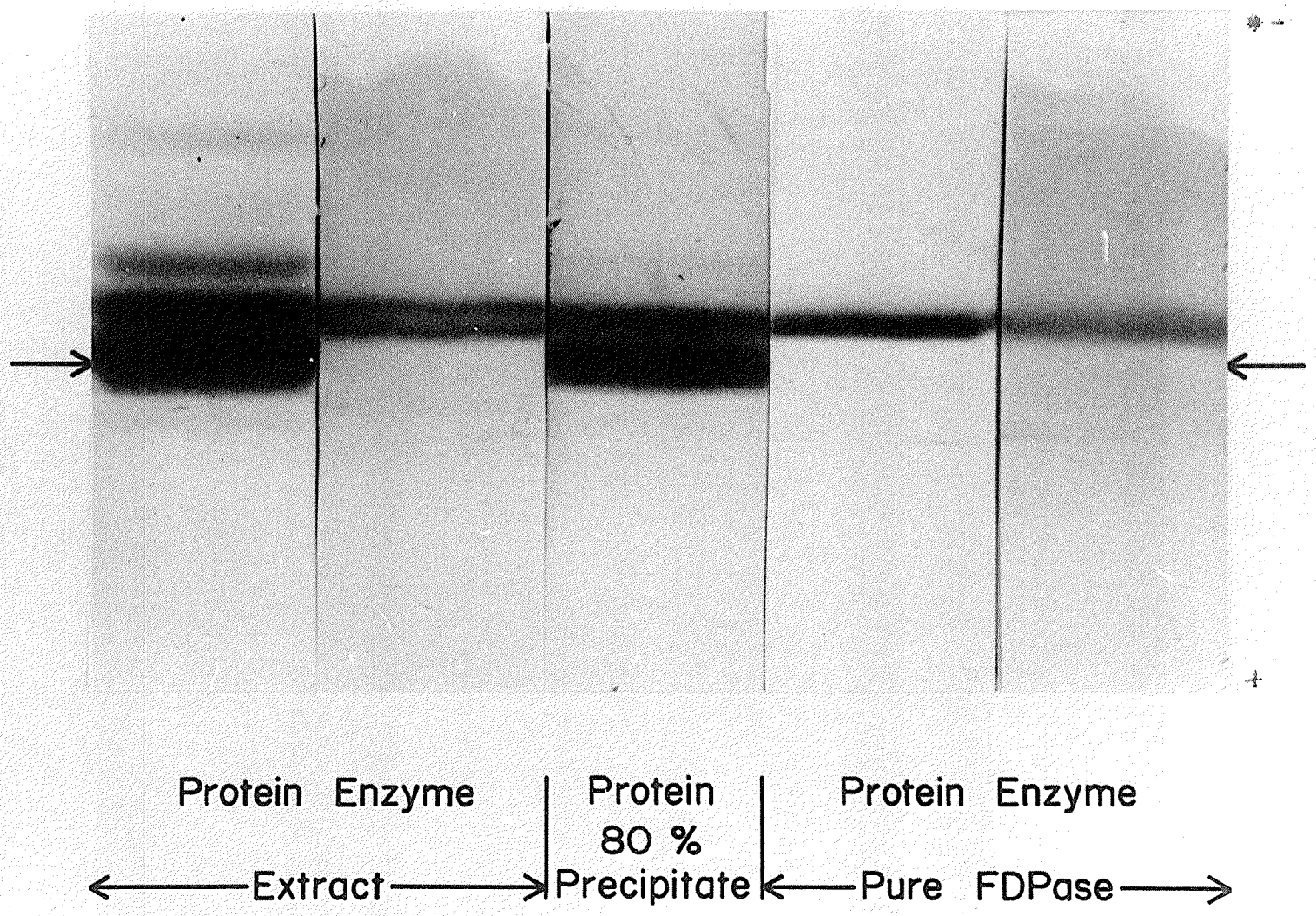
TABLE II

Purification of Chicken Breast Muscle FDPase

Fraction		Total enzyme activity	Yield (%)	Specific activity (units/mg protein)	Purification (fold)
I	Extract	5721	100.0	0.17	1.0
II	Ammonium sulphate precipitate (65-80%)	1196	20.9	0.64	3.9
III	Phosphocellulose eluate	466	8.2	23.41	141.0
IV	Sephadex G-200 eluate	----	-----	22.50	135.2

Figure 5

Electrophoresis of proteins present in purification fractions. Approximately 3-6 µl of each fraction was applied to the various strips. Electrophoresis was performed in 27 mM phosphate 2.7 mM EDTA-1 mM 2-mercaptoethanol, pH 6.0, at 5°C on 2.5 x 17 cm cellulose acetate strips (Gelman) at 300 V for 90 min with the origin equidistant from the electrodes. The strips for each fraction were stained for protein with Ponceau S. Strips for the extract and pure enzyme were stained for FDPase activity as described in Materials and Methods.


Protein Enzyme
Protein
80 %
Precipitate
Protein Enzyme
Extract
Pure FDPase

was the same in two separate purification experiments.

Often contaminants in an enzyme preparation are other enzymes which are, metabolically, closely related. FDPase from Step VI was assayed for 6 enzymes which utilize FDP or its immediate products and for two other more distantly related enzymes. Percentage contamination is expressed as the ratio of activity of the contaminating enzyme to that of FDPase, using the assays described in Materials and Methods. The assayed enzymes and the percentage contamination were: aldolase, less than 0.002%; glyceraldehyde 3-phosphate dehydrogenase, less than 0.002%: glycerophosphate dehydrogenase, less than 0.001%; glucose 6-phosphate dehydrogenase, less than 0.001%; phosphohexose isomerase, less than 0.017%; malic enzyme, less than 0.013%; and lactate dehydrogenase, less than 0.001%.

Anti-serum to chicken liver FDPase 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 the crystalline enzyme and this line fused with the single precipition line that developed with chicken liver extract (Fig. 6). The presence of a single precipitin band with the extract suggests that the enzyme was pure. For comparative purposes, rabbit liver extract was also included; it did not cross-react with the anti-serum.

The sedimentation velocity pattern of chicken liver FDPase is shown in the lower portion of Figure 7. No obvious extraneous slow or fast peaks or asymmetries,

Figure 6

Double diffusion analysis of chicken liver FDPase. Wells denoted F contained 2.4 mg of crystalline enzyme (equivalent to 33 units) per milliliter. Wells denoted C contained chicken liver extract (1.3 units / ml) while those denoted R contained rabbit liver extract (1.1 units / ml). The centre well contained anti-liver FDPase. The halos around the wells containing the extract are simply denatured protein. Conditions are described in Materials and Methods.

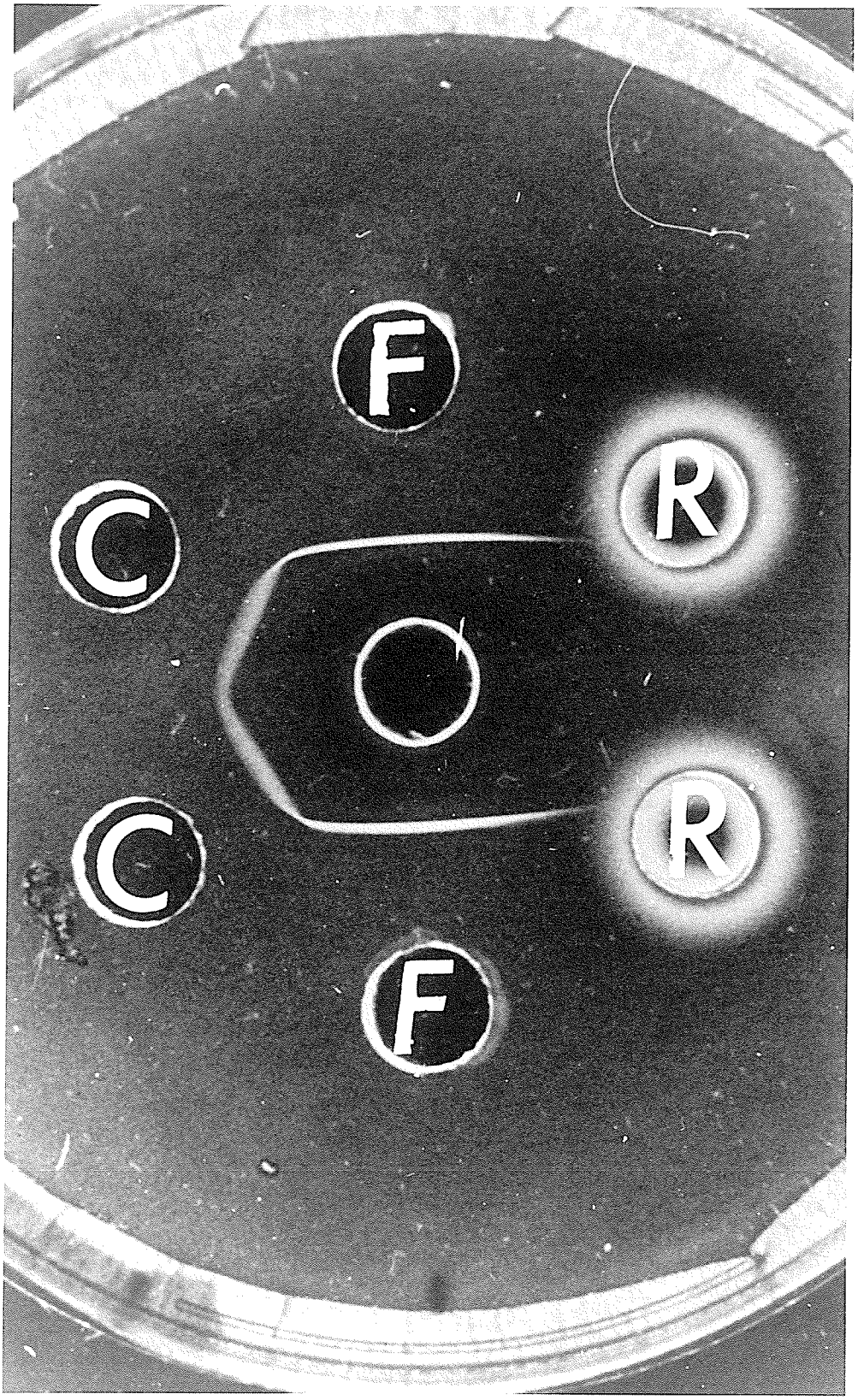
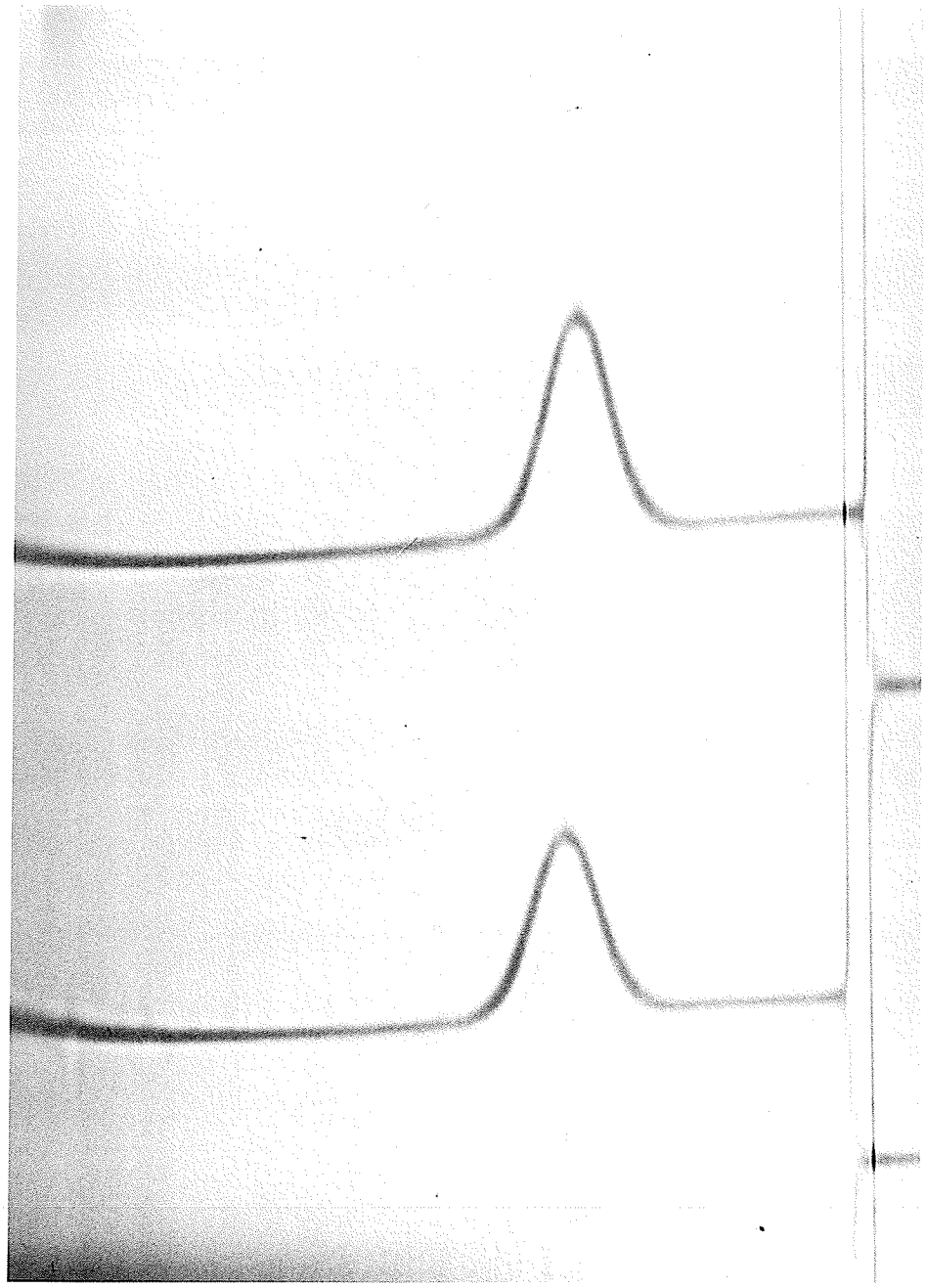

F
R
C
C
R
F

Figure 7

Sedimentation velocity patterns of avian FDPases. Upper peak, chicken breast muscle FDPase (4.7 mg/ml); lower peak, chicken liver FDPase (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.



which are indicative of contaminating proteins or denatured aggregates of liver FDPase were revealed. The crystalline form of chicken liver FDPase is given in Figure 8.

Criteria of Purity for Chicken Breast Muscle Fructose 1,6-Diphosphatase

Chicken breast muscle FDPase was tested for homogeneity by four methods: (a) purification to constant specific activity, (b) cellulose acetate electrophoresis, (c) absence of other enzymatic activities, and (d) ultracentrifugal analysis.

The enzyme retained constant specific activity following chromatography on phosphocellulose and Sephadex G-200 (Table II). The slightly lower specific activity following Sephadex G-200 chromatography may have been due to the presence of trace amounts of denatured proteins.

The pure enzyme appeared homogeneous when subjected to cellulose acetate electrophoresis at pH 6.0 and pH 8.8 (Fig. 9). In both cases, the protein remained as a single band with no detectable contamination.

Chicken breast muscle FDPase was also assayed for enzyme contaminants. The assayed enzymes and the percentage contamination were: aldolase, less than 0.050%; triose phosphate isomerase, less than 0.040%; glucose 6-phosphate dehydrogenase, less than 0.020%; phosphohexose isomerase, less than 0.070%; lactate dehydrogenase, less than 1.000%; malic enzyme, less than

Figure 8

Crystalline FDPase from chicken liver in an ammonium sulfate suspension (ph 6.0 at 7.0°C). The pictures were taken with bright-field objectives on a Zeiss photomicroscope under 63-fold magnification.

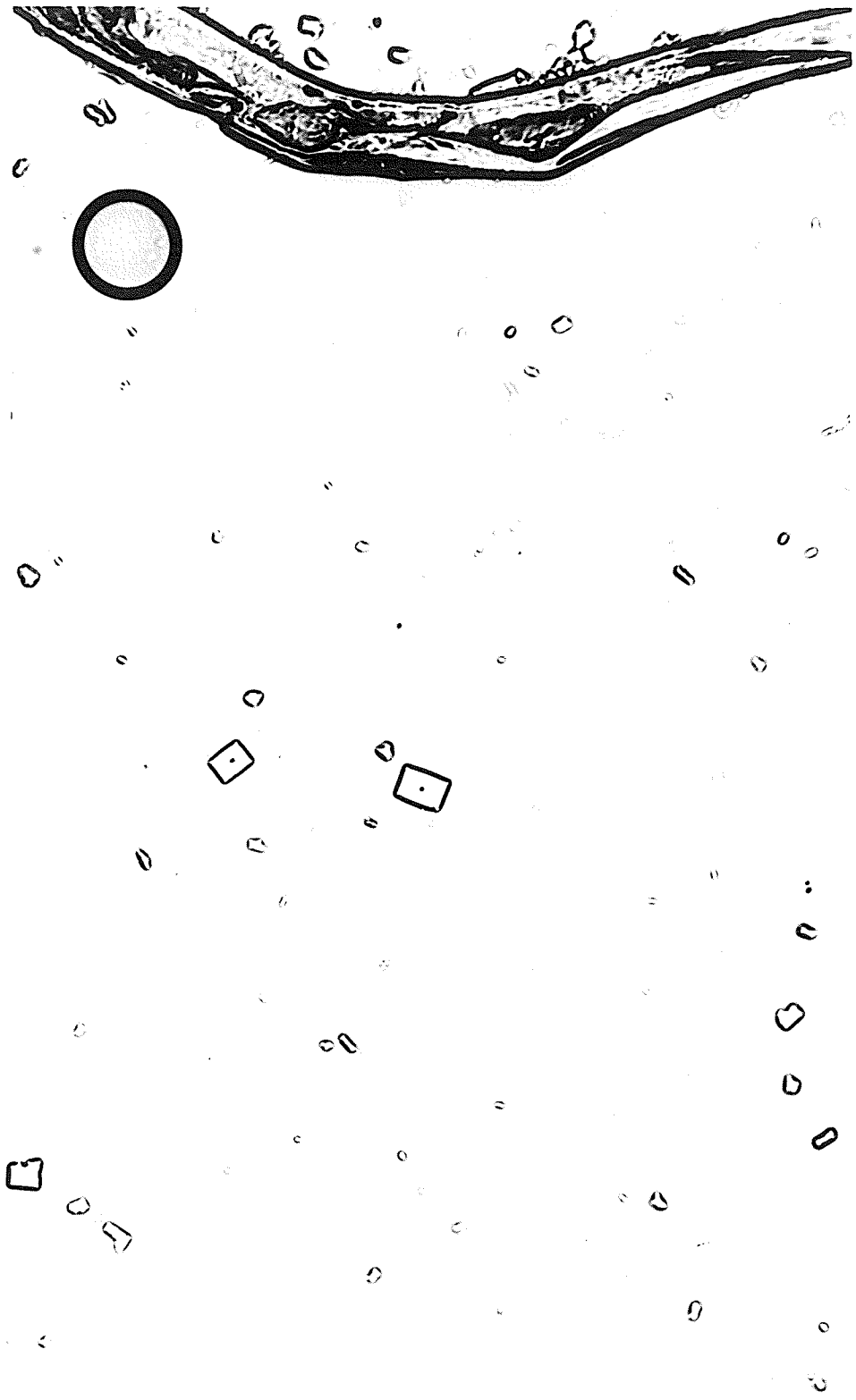


Figure 9

Electrophoresis of chicken breast muscle FDPase at two different pH's. Electrophoresis was performed in the pH 8.8 Gelman buffer as described in Fig. 4 and in the pH 6.0 phosphate buffer as described in Fig. 5, at 5°C on cellulose acetate strips. Approximately 70 µg of protein were applied to each strip. See Fig. 4 and Materials and Methods for additional details.

8.8 Hd 0.9 Hd

0.000%; and pyruvate kinase, less than 0.065%.

The sedimentation velocity pattern of chicken breast muscle FDPase is shown in the upper portion of Figure 7. Only a single symmetrical peak developed, which suggested that there was only one molecular species.

C. Molecular Properties of Avian Fructose 1,6-Diphosphatases

In order to be able to estimate the degree of molecular divergence of the chicken liver and chicken breast muscle FDPases, several studies were carried out to elucidate the particular molecular properties of each enzyme. The molecular properties determined were the electrophoretic mobilities; immunological cross-reactivity of anti-serum to chicken liver FDPase with the FDPases of livers of several species; isoelectric points; amino acid compositions; and molecular weights.

Electrophoretic Mobilities

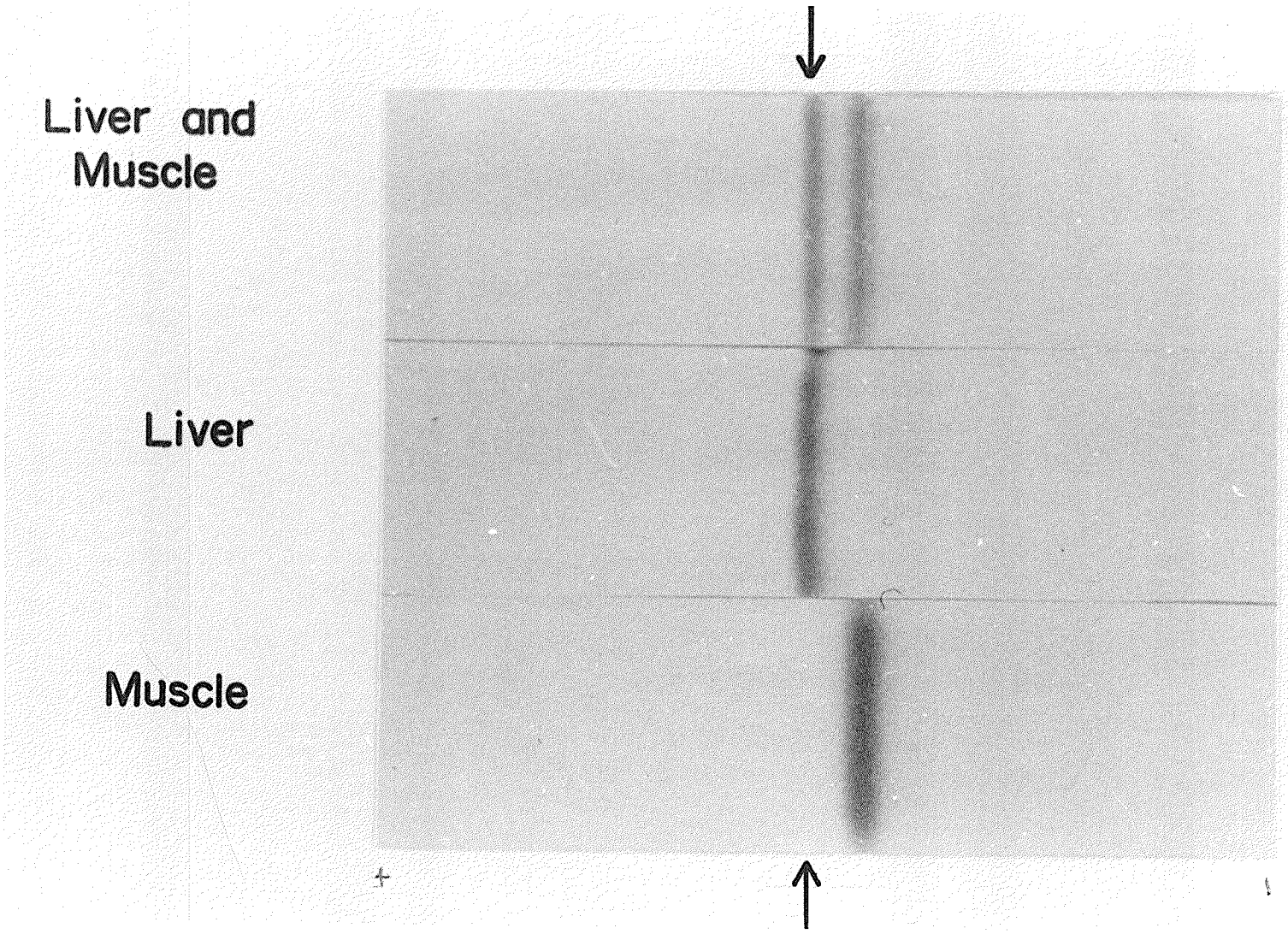
Electrophoresis was carried out on purified chicken liver and chicken breast muscle FDPases at pH 8.8. The two proteins exhibited different mobilities, even when mixed (Fig. 10).

Immunological Analysis

Immunological analysis of avian FDPases and the liver FDPases of several species were performed using anti-chicken liver FDPase. The analysis was carried out by means of double diffusion on agar plates and

Figure 10

Electrophoresis of chicken liver and breast muscle FDPases. Approximately 3-6 µl of each protein (or protein mixture) was applied to the various strips. Electrophoresis was performed in Gelman barbital - Tris salt mixture (17.8 g/l) - 1 mM EDTA - 1 mM 2-mercaptoethanol, pH 8.8, at 5°C on 2.5 x 17 cm cellulose acetate strips (Gelman) at 300 V for 90 min. with the origin equidistant from the electrodes. The strips for each protein (or protein mixture) were stained for protein with Ponceau S.


Liver and
Muscle
Liver
Muscle

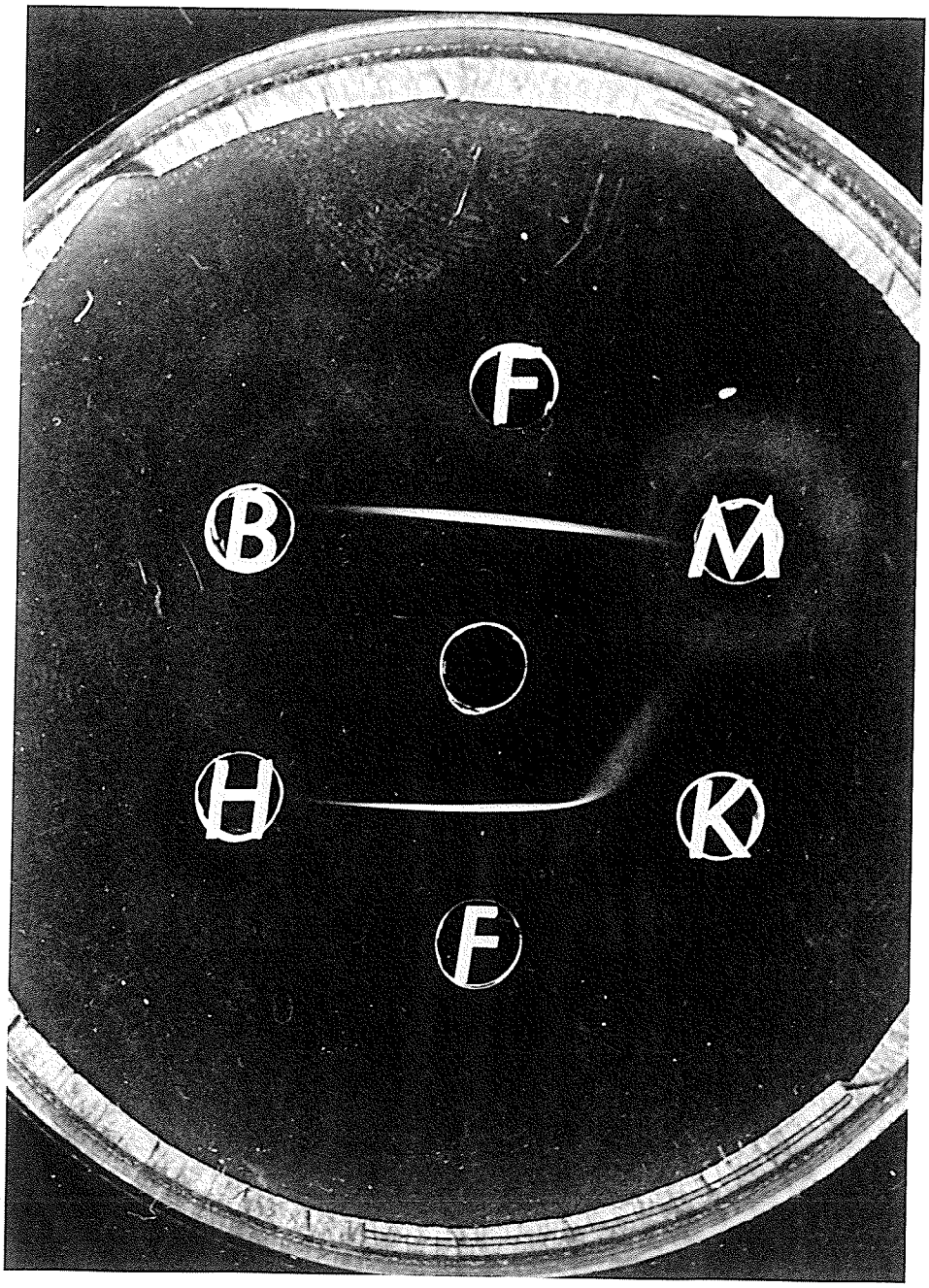
quantitative precipitin tests.

Using pure chicken liver FDPase, double diffusion range finding studies were conducted and optimum antigen concentrations were found in the region of from 0.68 to 2.72 mg/ml (9.32 to 37.26 units/ml). Detectable reactions, however, were observed at concentrations as low as 0.04 mg/ml (0.55 units/ml). A double diffusion analysis (Fig. 6) of anti-chicken liver FDPase against pure chicken liver FDPase (33 units/ml), chicken liver extract (1.3 units/ml), and rabbit liver extract (1.1 units/ml) illustrated the range of antigen concentration for antibody activity against the chicken liver FDPase. The lack of activity against the rabbit liver extract FDPase, which was at the same concentration as the chicken liver extract FDPase, is noteworthy. This is a rough indication of the degree of the specificity of the antibody——antigen reaction.

The results of double diffusion on agar plates of anti-chicken liver FDPase against the FDPases of chicken kidney, brain, heart muscle, and breast muscle extracts are shown in Figure 11. These results demonstrated the cross-reaction between anti-chicken liver FDPase and the pure liver enzyme (1.35 mg/ml) and the FDPase of kidney extract (3.1 units/ml). However, no cross-reaction was obtained between the anti-chicken liver FDPase and the FDPases of brain extract (0.45 units/ml), heart muscle extract (0.03 units/ml) or breast muscle extract

Figure 11

Double diffusion analysis of crystalline chicken liver FDPase (F) and the FDPases of chicken kidney (K), breast muscle (M), brain (B), and heart muscle (H). The centre well contained anti-chicken liver FDPase. Conditions are described in Materials and Methods.

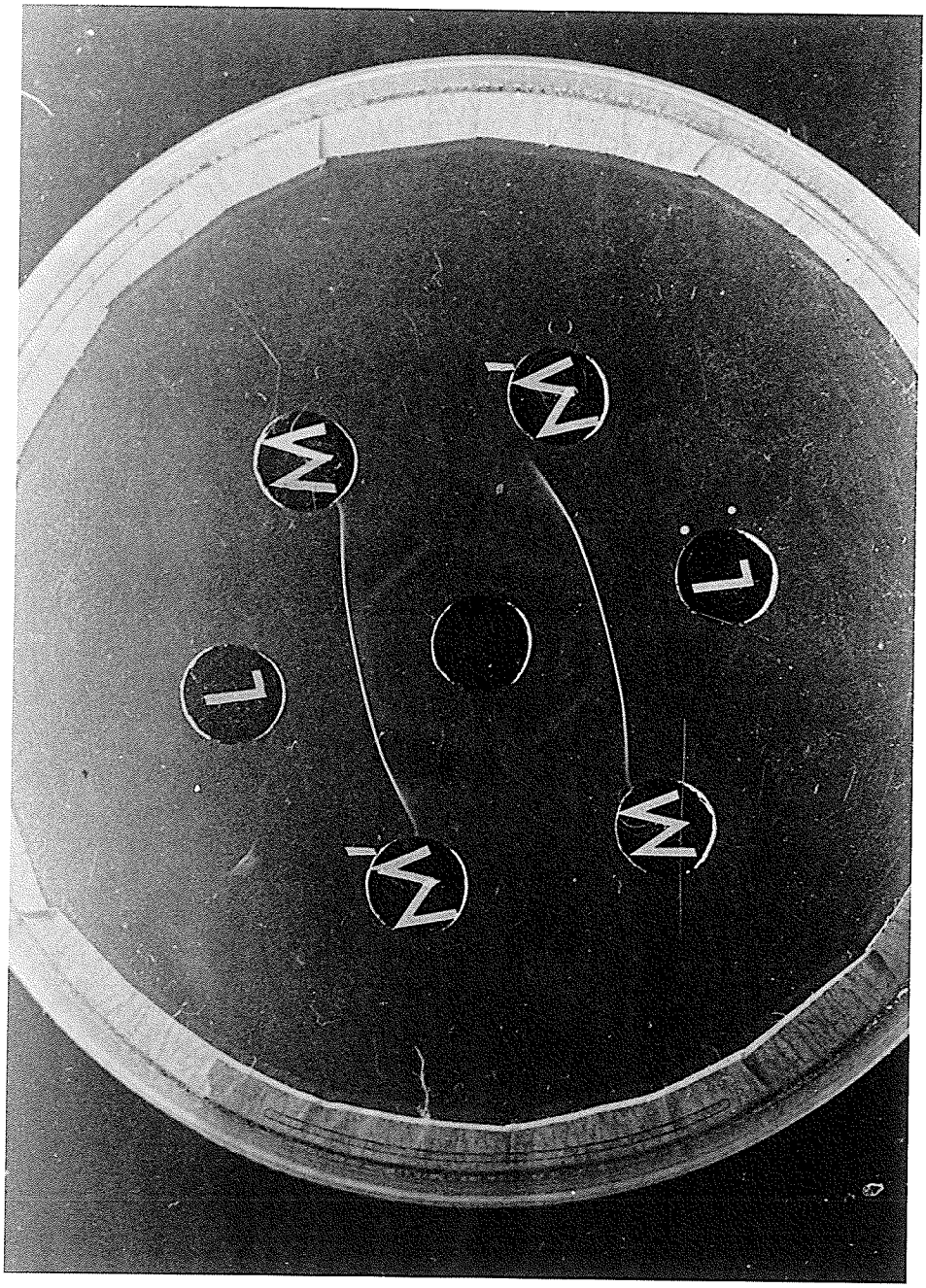

F
B
M
H
K
F

(1.7 units/ml). In these studies equal amounts of buffer (100 mM Tris-50 mM EDTA-10 mM 2-mercaptoethanol-100 mM KCl, pH 7.5 at 30°C) and tissue were homogenized. The low FDPase activities in the extracts were due to the low concentrations of the enzyme in the tissue extracts. In a later study, continuity between the precipitin bands of pure chicken liver FDPase (0.68 to 1.36 mg/ml), chicken liver extract FDPase (4.65 units/ml), and chicken kidney extract FDPase (3.95 units/ml) was demonstrated. In another experiment, a strong cross-reaction was obtained between anti-chicken liver FDPase and the liver enzyme (1.17 to 2.34 mg/ml) but comparatively little cross-reaction was obtained between the antibody and the breast muscle FDPase (2.34 to 4.67 mg/ml) (Fig. 12). The precipitin bands, formed by the antibody and the chicken liver and chicken breast muscle FDPases, showed spur formation. This was indicated by a lack of continuity between the precipitin bands. In this experiment, the concentration of breast muscle FDPase was much higher than that used in Figure 11. This explains the lack of precipitin bands against breast muscle FDPase in Figure 11.

By means of an indirect immunological titration of anti-chicken liver FDPase with pure chicken liver FDPase and extract preparations of chicken liver, kidney, and breast muscle, it was possible to determine quantitatively the extent of cross-reaction. A constant amount of

Figure 12

Double diffusion analysis of the pure FDPases of chicken liver and chicken breast muscle. Wells denoted L and L' contained 2.3 and 1.2 mg/ml of crystalline liver FDPase, respectively. Wells denoted M and M' contained 4.7 and 2.3 mg/ml of pure breast muscle FDPase, respectively. The centre well contained anti-chicken liver FDPase. Conditions are described in Materials and Methods.


N
N
L
L
N
N

enzyme or extract preparation (0.34 units/ml) was added to doubly diluted amounts of anti-chicken liver FDPase. After incubation and centrifugation, the supernatants were assayed for residual antigen by measuring the FDP cleavage activity in the supernatant. The results shown in Figure 13 demonstrate that anti-liver FDPase inhibited all of the FDPase activity in the pure enzyme and that of chicken liver and kidney extracts. However, it had much less effect on the FDPase activity of the chicken breast muscle extract. Undiluted anti-chicken liver FDPase gave an 84% inhibition of chicken breast muscle FDPase activity.

Anti-chicken liver FDPase was used in several immunochemical analyses of the liver FDPases of several species. In the double diffusion on agar plates of anti-chicken liver FDPase against crystalline liver FDPase (1.35 mg/ml) and the FDPases of liver extracts of rat (5.9 units/ml), rabbit (3.8 units/ml), budgerigar (5.4 units/ml), and turkey (8.2 units/ml), there was good cross-reaction between the anti-serum and the crystalline chicken liver FDPase and the liver FDPases of budgerigar and turkey (Fig. 14). The continuity between the precipitin bands of chicken liver FDPase and turkey liver extract FDPase suggested a fairly high degree of similarity between the two enzymes. However, spur formation between the precipitin bands of budgerigar liver extract FDPase and chicken and turkey liver FDPases suggested

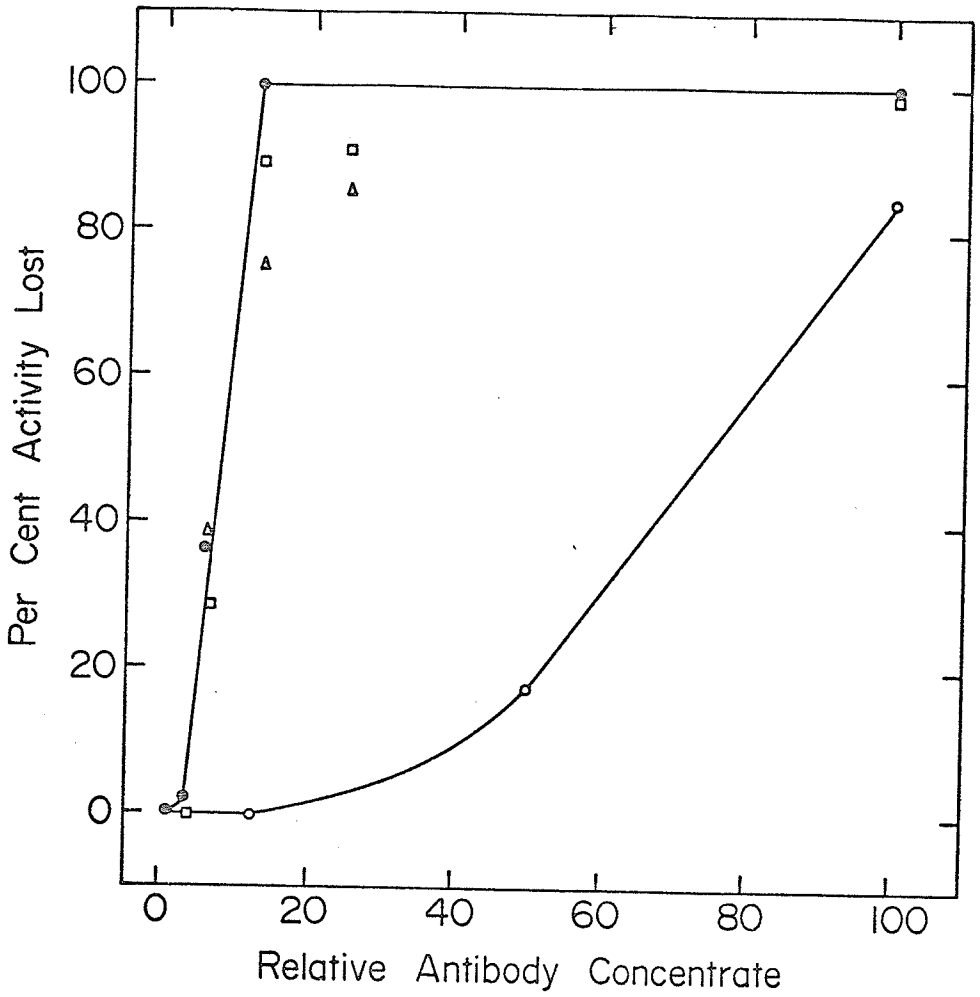
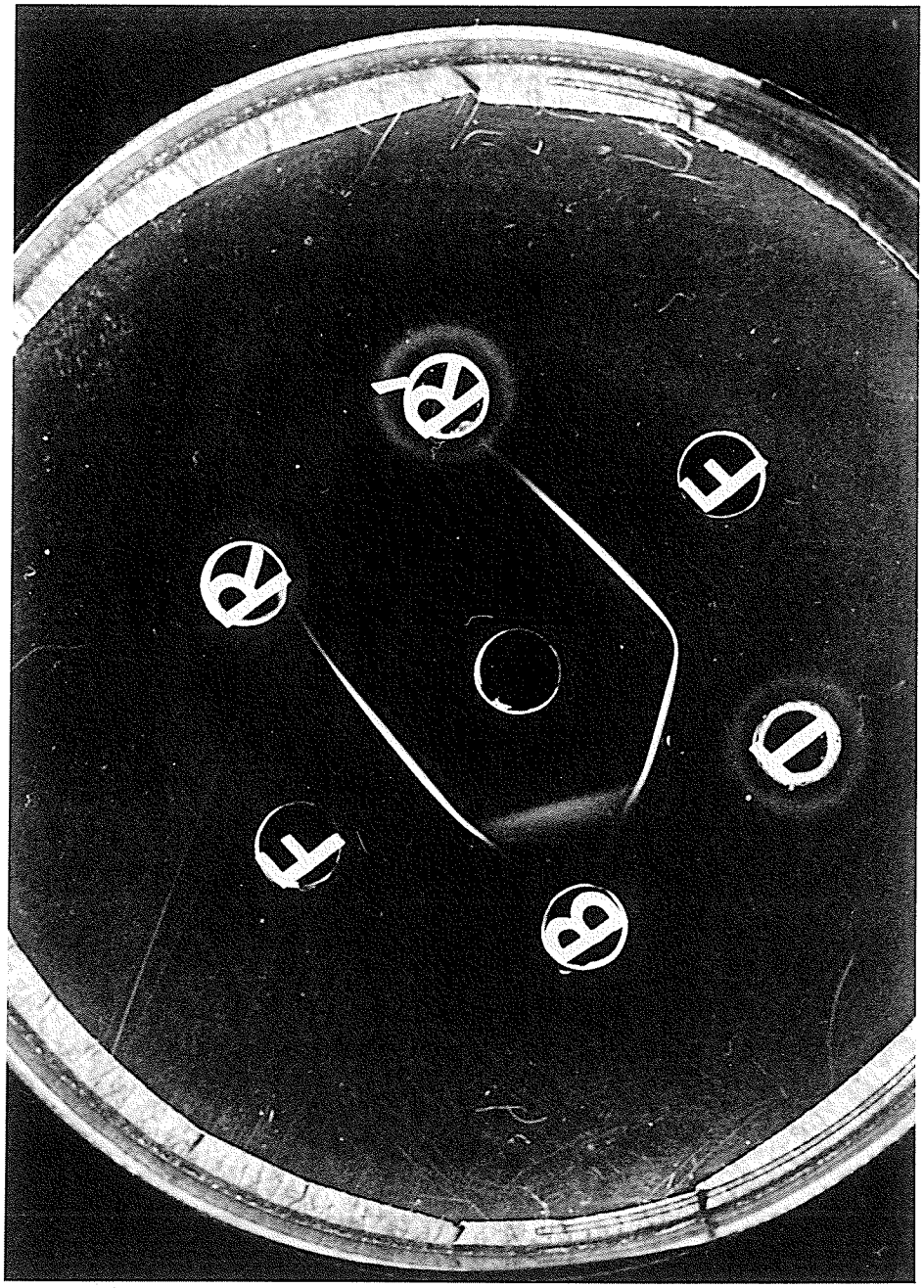


Figure 13

Precipitation of crystalline chicken liver FDPase (●) and the FDPases of extracts of chicken liver (□), kidney (△), and breast muscle (○) with anti-serum to chicken liver FDPase. 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 x g for 20 min and FDPase was assayed in each filtrate.

Figure 14

Double diffusion analysis of crystalline chicken liver FDPase (F) and the liver FDPases of rabbit (R^1), rat (R), budgerigar (B), and turkey (T). The centre well contained anti-chicken liver FDPase. Conditions are described in Materials and Methods.


A
B
C
D
E
F

that budgerigar liver FDPase was immunologically different from the FDPases of chicken and turkey livers. There was, however, little or no cross-reaction with the liver FDPases of rat and rabbit.

In the quantitative precipitation of the anti-serum to chicken liver FDPase with crystalline liver enzyme and the FDPases of rat, rabbit, budgerigar, and turkey liver extracts (0.19 units/ml), variations were found in the extent of cross-reaction between anti-chicken liver FDPase and the liver extracts of the various species (Fig. 15). The extent of cross-reaction was about the same for chicken and turkey liver FDPases and somewhat less for budgerigar liver FDPase. Cross-reactions between the anti-serum and the liver FDPases of rat and rabbit were least. At high anti-serum concentrations, approximately 71 and 16% of the activities of these latter two enzymes were lost due to precipitation.

Isoelectric Points

In both determinations of the isoelectric point of chicken liver FDPase, the enzyme exhibited homogeneity in that only one protein absorbance peak was found in the fractions. This was coincident with the presence of a single FDPase activity peak. Liver FDPase showed an isoelectric point of pH 7.8 using the ampholyte pH range of 3 to 10 and pH 8.1 using the ampholyte pH range of 7 to 10 (Fig. 16). An extraneous absorbance peak was found in the latter determination at pH 7.2; this was non-protein in nature.

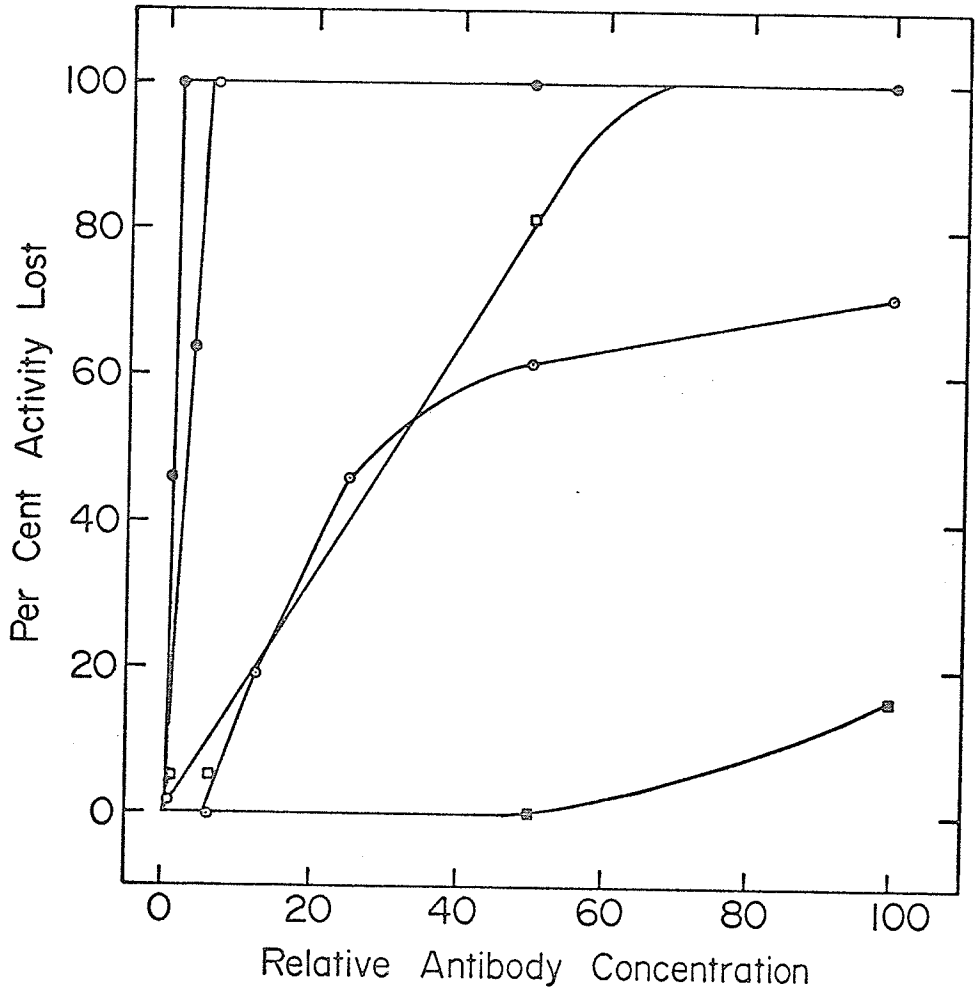


Figure 15

Precipitation of crystalline chicken liver FDPase (○) and the liver FDPases of turkey (●), budgerigar (□), rat (⊙) and rabbit (▣) with anti-serum to chicken liver FDPase. 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 (2.5 units/ml) or tissue extract (2.5 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 x g for 20 min and FDPase was assayed in each filtrate.

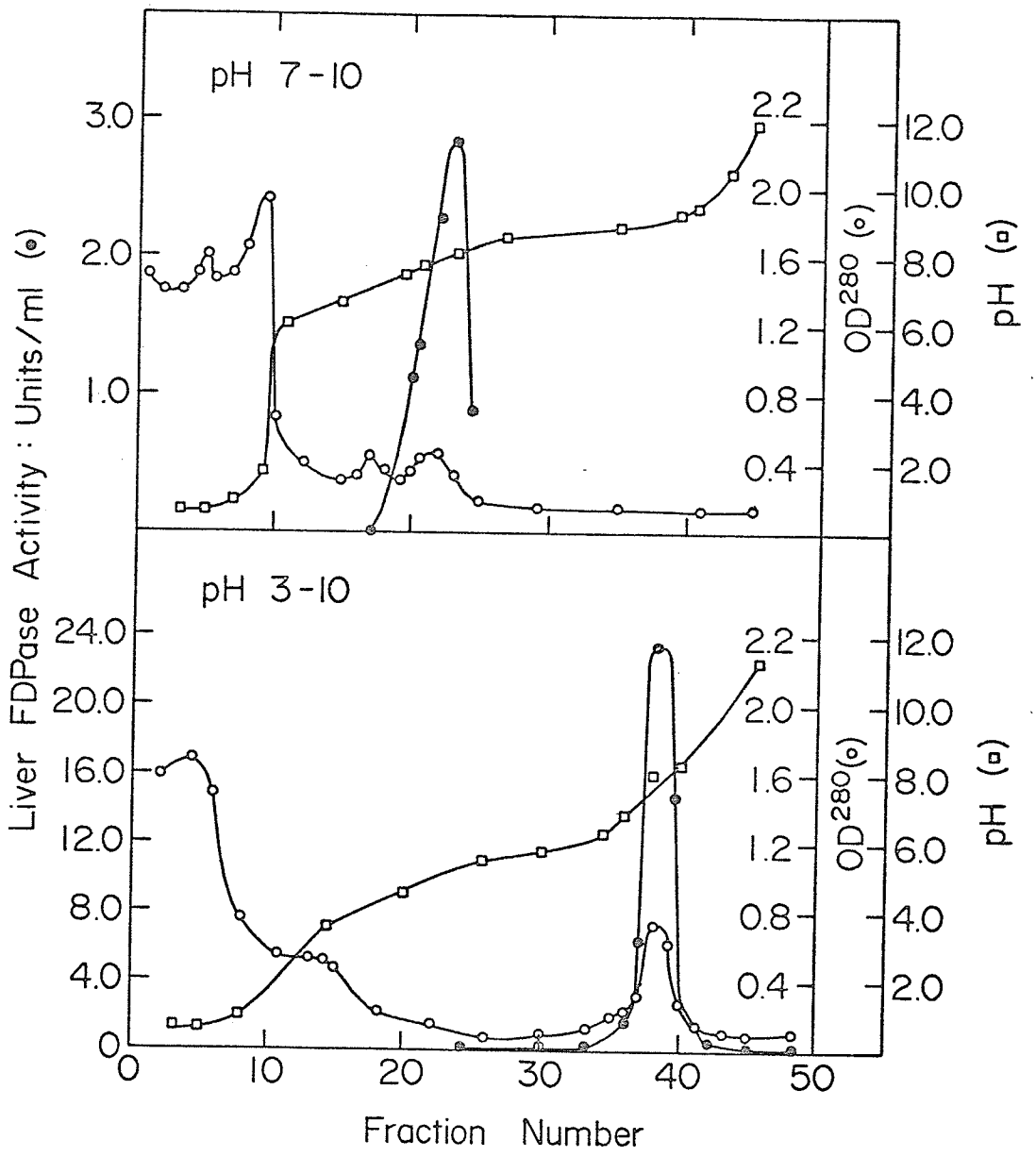


Figure 16

Profiles of isoelectric point determination of liver FDPase. Electrofocusing of the enzyme at two different pH ranges was carried out as described in Materials and Methods.

In the determination of the isoelectric point of breast muscle FDPase, the enzyme also exhibited homogeneity in that only one protein absorbance peak was found in the fractions. This was coincident with the presence of a single FDPase activity peak. Muscle FDPase showed an isoelectric point of pH 8.4 using the ampholyte pH range of 3 to 10 and pH 8.5 using the ampholyte pH range of 7 to 10 (Fig. 17).

Amino Acid Composition

Amino acid analyses of chicken liver FDPase and chicken breast muscle FDPase were performed on each duplicate 24-, 48-, and 72- h set of hydrolysates (Tables III and IV). Of the total dry matter hydrolyzed, 98% of the liver FDPase and 101% of muscle FDPase were recovered as amino acids. The nitrogen content of chicken liver FDPase, as estimated from the amino acid composition, was 16.8% while that of chicken breast muscle FDPase, was 16.7%.

Both enzymes were corrected for threonine and serine decomposition and ammonium formation from amino acids by extrapolation to zero time. With the liver enzyme, the valine yield increased 7 % between the 24- and 48- h hydrolysates while the 48- and 72-h values remained relatively constant. The latter values were used to represent valine. The isoleucine yield increased with each successive hydrolysis period; the 72-h value was used to represent isoleucine. In the case of muscle

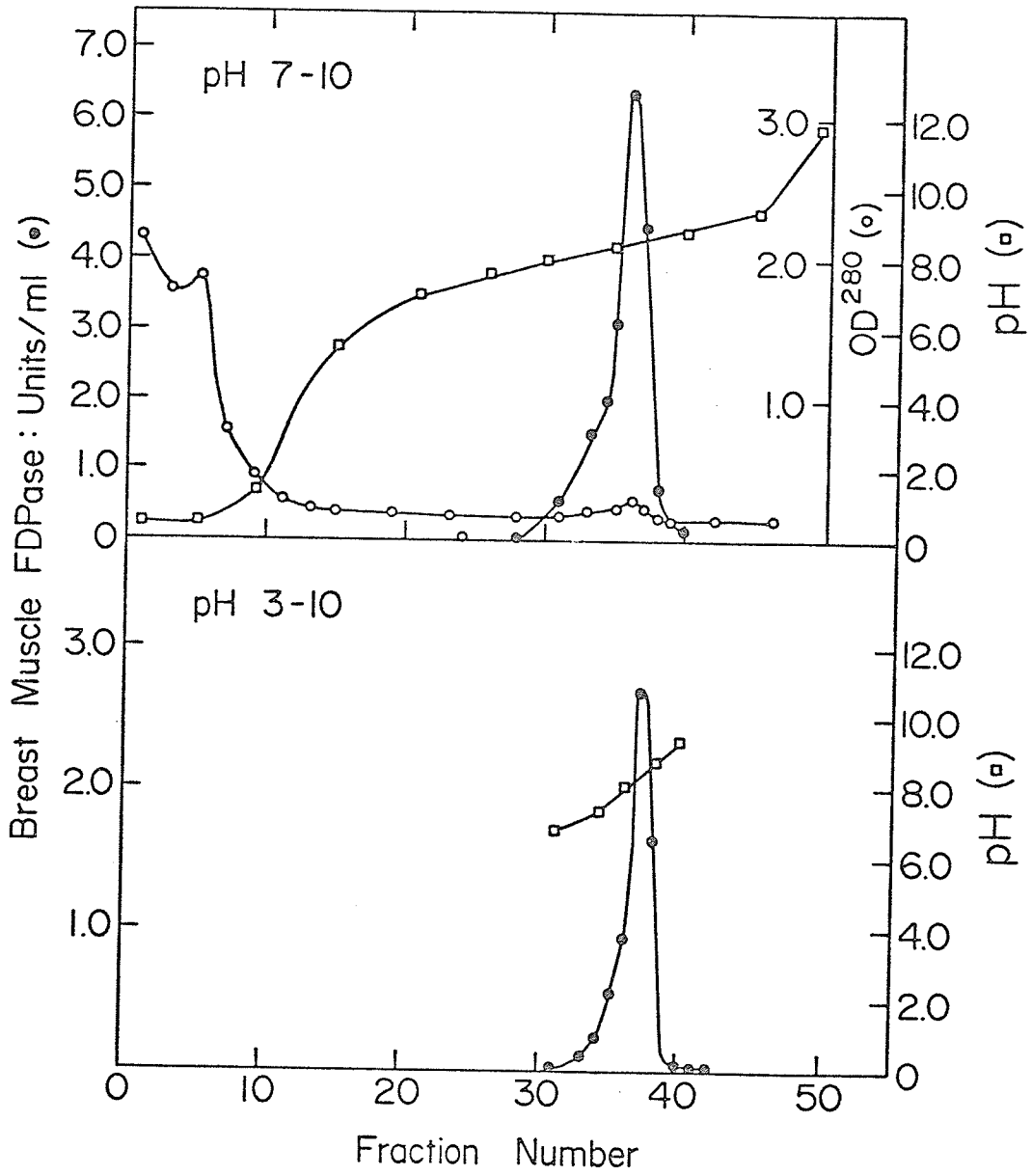


Figure 17

Profiles of isoelectric point determination of breast muscle FDPase. Electrofocusing of the enzyme at two different pH ranges was carried out as described in Materials and Methods.

TABLE III

Amino Acid Composition of Chicken Liver FDPase

Amino Acids	Quantity hydrolyzed (umoles) after[a] 24h	48h	72h	Average	Number of amino acids per 135,000 g	Nearest integral number
Lys	0.188	0.187	0.191	0.189	99.1 ± 0.6[h]	99
His	0.027	0.028	0.027	0.027	14.2 ± 0.5	14
Arg	0.107	0.106	0.104	0.106	55.6 ± 3.5	56
Asp	0.259	0.257	0.271	0.262	137.4 ± 2.3	137
Thr	0.123	0.120	0.114	0.130[b]	68.2 ± 4.4	68
Ser	0.144	0.131	0.116	0.158[b]	82.9 ± 0.8	83
Glu	0.154	0.157	0.156	0.156	81.8 ± 2.4	82
Pro	0.087	0.090	0.082	0.086	45.1 ± 1.4	45
Gly	0.221	0.218	0.216	0.218	114.3 ± 2.3	114
Ala	0.196	0.198	0.193	0.196	102.8 ± 0.7	103
Cys				0.039[c]	20.5 ± 0.6	20
Val	0.193	0.207	0.204	0.206[d]	108.1 ± 4.1	108
Met	0.077	0.039	0.038	0.039[d]	20.5 ± 5.3	20
Ile	0.155	0.169	0.178	0.178[e]	93.4 ± 1.1	93
Leu	0.196	0.194	0.199	0.196	102.8 ± 1.6	103
Tyr	0.097	0.091	0.086	0.103[b]	54.0 ± 0.1	54
Phe	0.078	0.078	0.079	0.078	40.9 ± 1.9	41
Try				0.004[f]	2.1 ± 0.4	2
NH_3	0.377	0.373	0.363	0.371[g]		

a Each time interval value represents the average of two or more determinations.

b Extrapolated to zero time.

c Value obtained from sulfhydryl titration using Ellman's reagent (16).

d Average of 48 - and 72 - h hydrolysis.

e Value of 72-h hydrolysis.

f Determined spectrophotometrically by method of Edelhoch (13).

g Value corrected for ammonia obtained following chromatography of 24-h blank and decomposition of threonine, serine, and tyrosine.

h ± indicates standard deviation.

TABLE IV

Amino Acid Composition of Chicken Breast Muscle FDPase

Amino Acids	Quantity hydrolyzed (umoles) after[a] 24h	48h	72h	Average	Number of amino acids per 135,000 g	Nearest integral number
Lys	0.163	0.159	0.174	0.165	111.9 ± 0.9[g]	112
His	0.025	0.029	0.034	0.029	19.7 ± 0.3	20
Arg	0.062	0.053	0.065	0.060	40.7 ± 0.4	41
Asp	0.151	0.159	0.163	0.158	107.2 ± 3.8	107
Thr	0.109	0.108	0.105	0.113[b]	76.7 ± 3.3	77
Ser	0.108	0.099	0.092	0.116[b]	78.7 ± 5.7	79
Glu	0.178	0.176	0.186	0.180	122.1 ± 3.1	122
Pro	0.088	0.089	0.092	0.090	61.1 ± 0.3	61
Gly	0.166	0.167	0.168	0.167	113.3 ± 1.0	113
Ala	0.146	0.151	0.153	0.150	101.8 ± 0.9	102
Cys				0.029[c]	19.7 ± 1.3	20
Val	0.123	0.138	0.140	0.139[d]	94.3 ± 1.8	94
Met	0.018	0.020	0.023	0.020	13.6 ± 2.6	14
Ile	0.088	0.098	0.099	0.099[d]	67.2 ± 0.6	67
Leu	0.179	0.181	0.180	0.180	122.1 ± 0.2	122
Tyr	0.074	0.073	0.072	0.073	49.5 ± 1.3	50
Phe	0.062	0.061	0.059	0.061	41.4 ± 1.9	41
Try				0.004[e]	2.7 ± 0.5	3
NH_3	0.104	0.112	0.120	0.112[f]		

a Each time interval represents the average of two or more determinations.

b Extrapolated to zero time.

c Value obtained from sulfhydryl titration using Ellman's reagent (16).

d Average of 48-and 72-h hydrolysis.

e Determined spectrophotometrically by method of Edelhoch (13).

f Value corrected for ammonia obtained following chromatography of 24-h blank and decomposition of threonine and serine.

g ± indicates standard deviation.

FDPase, valine and isoleucine yields increased 6 and 15%, respectively, between the 24- and 48-h hydrolysates while the 48- and 72-h values remained fairly constant. The latter values were used to represent valine and isoleucine.

The number of sulfhydryl groups was estimated by the method of Ellman (16). In 8.5 M urea, the reactions of both enzymes were completed within several minutes; 6800 and 7040 µg of liver and muscle enzymes reacted with 1.0 µmoles of Ellman's reagent. This was equivalent to an -SH content of 0.146 µmoles /1.0 mg (20 cysteine residues per 135,000 g) of liver FDPase and 0.142 µmoles /1.0 mg (19 cysteine residues per 135,000 g) of muscle enzyme.

Tryptophan analysis by the method of Edelhoch (13) yielded a tryptophan content of 2.1 residues per 135,000 g of chicken liver FDPase. The method of Goodwin and Morton (25) yielded a tyrosine:: tryptophan ratio of about 48.5. This latter value would suggest that the liver enzyme did not contain tryptophan. Other researchers (11, 13) have also obtained similar discrepancies between the two procedures and have presented evidence that the Edelhoch procedure is more reliable. In this study the Edelhoch value was considered to be most representative of the true tryptophan content of chicken liver FDPase. The Edelhoch method also yielded a tryptophan content of 2.8 residues per 135,000 g of chicken breast muscle FDPase.

The partial specific volumes of chicken liver FDPase,

and chicken breast muscle FDPase, as estimated from the amino acid composition (12), were both 0.743 cm^3/g.

The dry matter contents of chicken liver FDPase and muscle FDPase, as estimated by the method of Walsh and Brown (90), were found to be 1.36 times the absorbance of the protein sample at 280 mμ for a 1 cm light path. Using the same method, the respective dry matter contents of the liver and muscle FDPases were found to be 0.86 and 0.78 times the protein content obtained with the procedure of Lowry et al. (35). Bovine serum albumin was used as the standard reference protein for the Lowry determinations.

Estimation of Molecular Weights

The method of Leach and O'Shea (34) was used to determine the molecular weight of chicken liver FDPase. The molecular weights of the reference proteins were: ovalbumin, 45,500 (3); bovine serum albumin, 70,000 (93); and rabbit muscle aldolase, 158,000 (27). Crystalline chicken liver FDPase was eluted at 1.69 times the void volume. From the same column under the same conditions, ovalbumin was eluted at 2.17, bovine scrum albumin at 1.99 and rabbit aldolase at 1.62 times the void volume. The elution position for chicken liver FDPase on the calibration corresponded to an apparent molecular weight of 134,900 $\pm$ 4400 (Fig. 18).

The Stokes radius of chicken liver FDPase was calculated from the Sephadex G-200 data (Fig. 19) according to the procedure of Ackers (1). Stokes radii of the

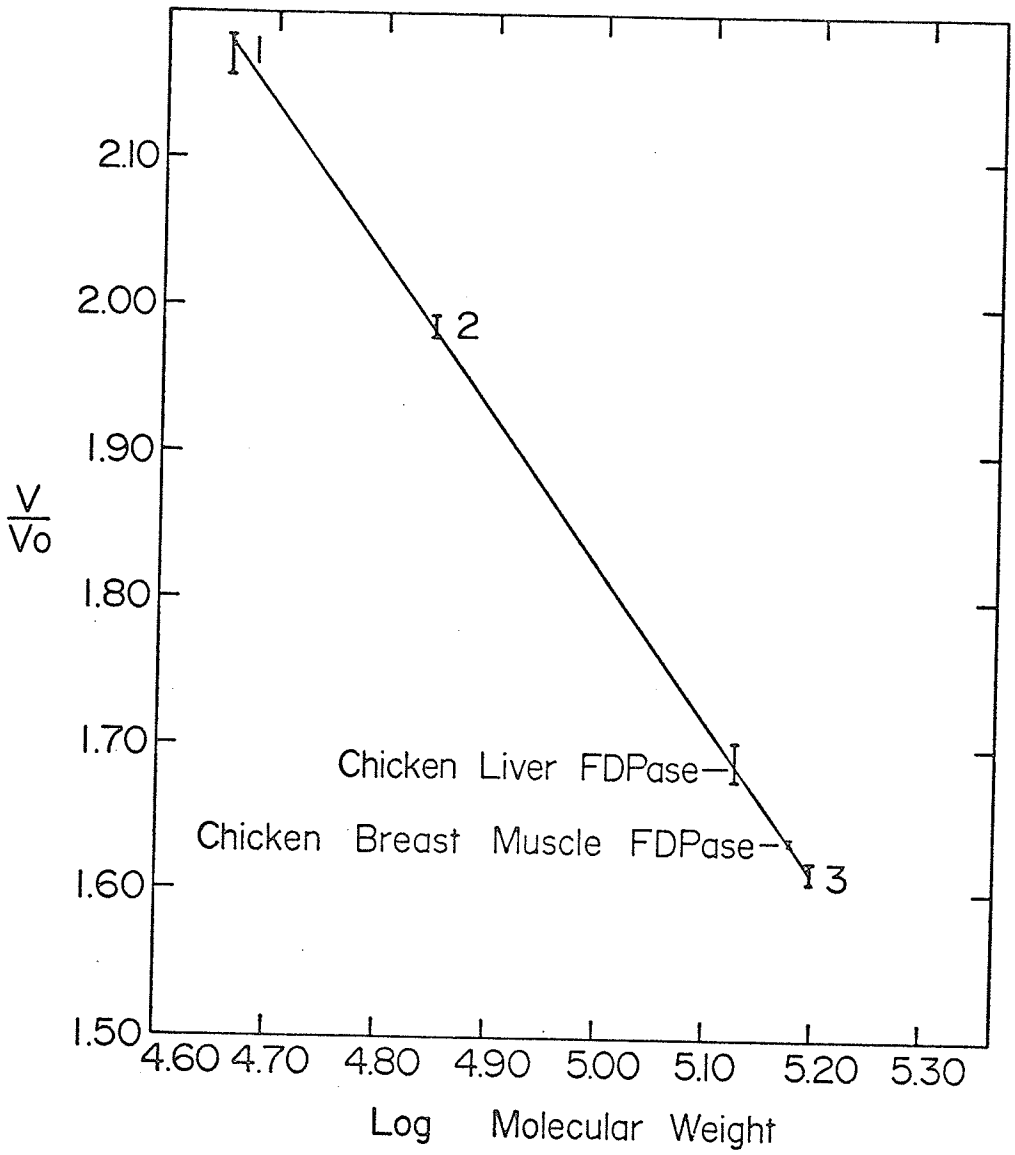


Figure 18

Logarithm of the molecular weight plotted against the ratio of the protein elution volume (V) to column void volume (Vo) at 24 ± 2°C. 1. ovalbumin; 2. bovine serum albumin; and 3. rabbit muscle aldolase. Duplicate elutions were carried out on all proteins. Standard deviations of the elution ratios (V/Vo) of the various proteins is indicated by I . See text for additional details.

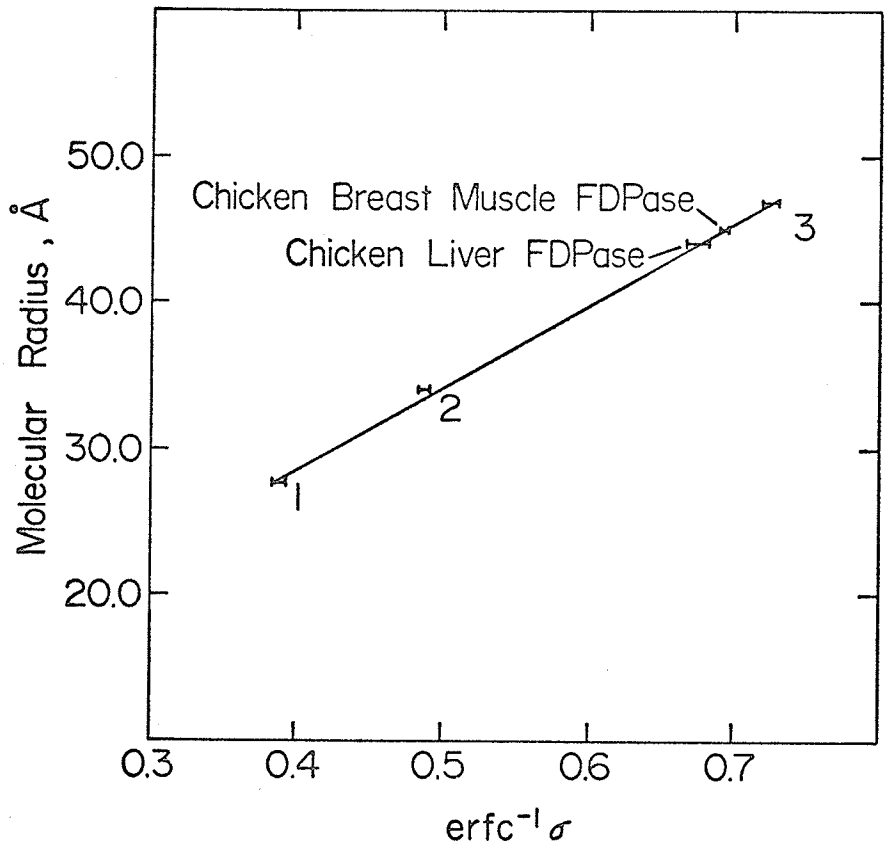


Figure 19

Determination of the molecular radius of chicken liver and muscle FDPases by calibration of a Sephadex G-200 column by plotting the Stokes radius of several reference proteins against the inverse error function complement of the partition coefficient according to the method of Ackers (1). 1. ovalbumin; 2. bovine serum albumin; 3. rabbit muscle aldolase. Standard deviations of the $erfc^{-1}\sigma$ of the various proteins is indicated by ⊢⊣ . See text for additional details.

standard reference proteins were: ovalbumin, 27.6 Å (14); bovine serum albumin, 34 Å (2); and rabbit muscle aldolase, 47 Å (39). The Stokes radius of chicken liver FDPase was found to be 43.9 Å.

The molecular weight of chicken breast muscle FDPase was determined as described previously. The molecular weight of chicken liver FDPase, which was used as a reference protein, was 135,000. Under the conditions of this experiment, chicken muscle FDPase was eluted at 1.68 times the void volume while chicken liver FDPase was eluted at 1.74 times the void volume. These elution ratios were corrected back to the elution ratio of chicken liver FDPase from the previous experiment and were then superimposed on the previous calibration curve. Using this method, the elution position for chicken breast muscle FDPase on the calibration curve corresponded to an apparent molecular weight of 152,050 $\pm$ 500 (Fig. 18).

The Stokes radius of chicken breast muscle FDPase was also calculated from the Sephadex G-200 data. A distribution coefficient was calculated for each of the chicken liver and breast muscle FDPases from their respective data. These values were corrected back to the distribution coefficient of chicken liver FDPase from the previous experiment and the inverse error function complement of the partition coefficient for chicken breast muscle FDPase was determined. Using this method, the position of chicken breast muscle FDPase on the calibration

curve corresponded to an apparent Stokes radius of 45 Å (Fig. 19).

Sedimentation velocity studies were carried out for chicken liver FDPase. Values of the sedimentation coefficient of the liver enzyme were determined at several protein concentrations and were corrected to the viscosity and density of water at 20°C. The value of $S_{20,w}$ obtained by extrapolation to zero protein concentration was 7.40×10^{-13} (Fig. 20). It was evident that the sedimentation coefficient is appreciably affected by protein concentration within the range studied.

The molecular weight of chicken liver FDPase estimated by combining the sedimentation coefficient ($S_{20,w}$) and the diffusion coefficient ($D_{20,w}$), according to the procedure of Svedberg (86), was 143,300. For all of the calculations, the value of the partial specific volume of chicken liver FDPase, which was calculated from the amino acid composition, was assumed to be 0.743 cm^3/g under all conditions.

Sedimentation velocity studies were also carried out for chicken breast muscle FDPase. However, the results were so variable that a sedimentation coefficient for the enzyme could not be determined, and hence, the molecular weight of chicken breast muscle FDPase could not be estimated by the above method.

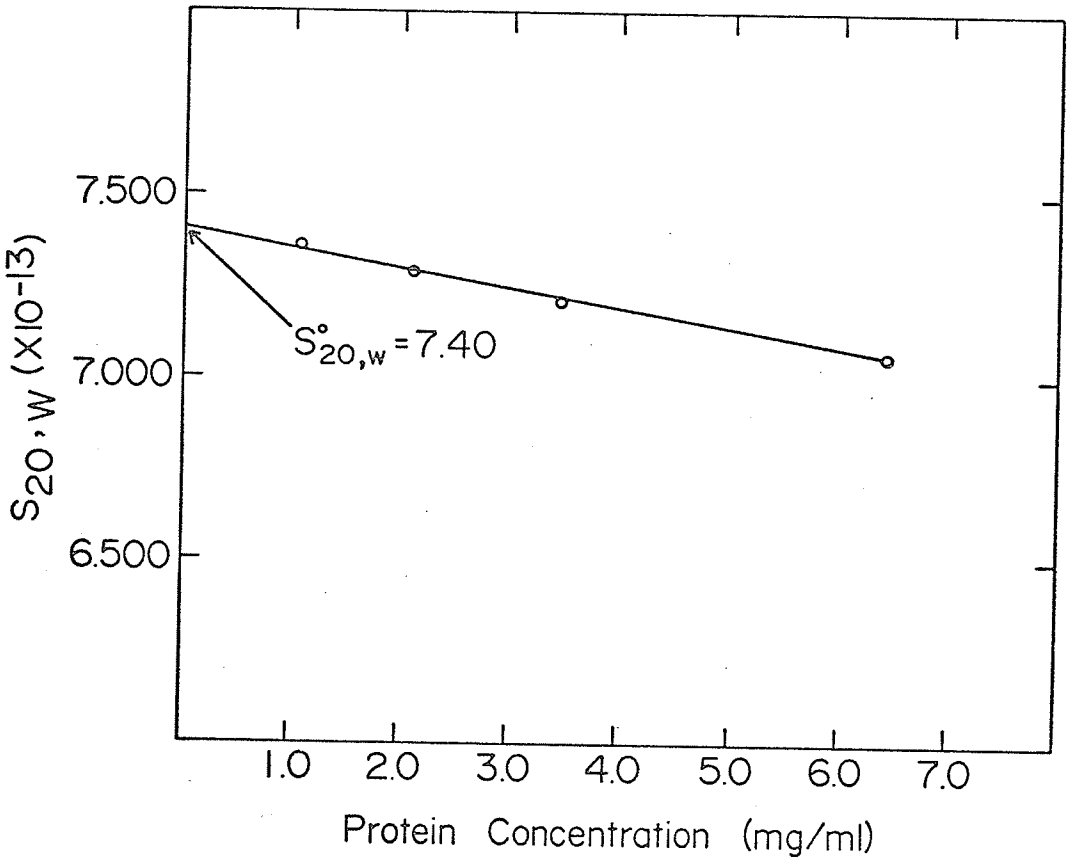


Figure 20

Extrapolation of the apparent sedimentation coefficients ($S_{20,w}$) of chicken liver FDPase to zero protein concentration. See text for further details.

Discussion

FDPase has been purified from chicken liver and chicken breast muscle. Both enzyme preparations were shown to be pure by a number of criteria.

Studies were carried out on several of the molecular properties of the pure enzymes. A comparison of these properties indicates that there are, at least, two isozymic forms of the enzyme; one present in chicken liver and kidney and another in chicken breast muscle. These comparisons are substantiated by the comparison of the molecular properties of the rabbit tissue FDPase isozymes (17, 18, 20, 31).

At pH 8.8, the electrophoretic mobilities of the pure liver and breast muscle FDPases are sufficiently different to cause complete separation of the two proteins when mixed (Fig. 10). This difference in mobility indicates differences in the charge distributions of the isozymes at pH 8.8. This, in turn, may be due to differences in the relative proportions of acidic and basic side chains of the constitutive amino acids of the two enzymes. Similar electrophoretic differences between the liver and muscle isozymes of rabbit have been reported by Enser *et al.* (17) and Fernando *et al.* (20).

The isoelectric points of chicken liver and breast muscle FDPases have been found to be from pH 7.8 to 8.1 (Fig. 16) and pH 8.4 to 8.5 (Fig. 17), respectively. The differences between the nature of the charges of these enzymes give support to the differences in their charge distributions, as initially indicated by their

electrophoretic mobilities at pH 8.8. It should be added that apparent discrepencies occurred between the direction of movement of the proteins during electrophoresis at various pH's and their isoelectric points. The liver FDPase showed no mobilities under conditions which, according to its isoelectric point, there should be a net charge on the molecule. The muscle FDPase exhibited a negative charge at pH 6.0 and a positive charge at pH 8.8. Based on the enzyme's isoelectric point, the direction of its mobility at these pH's should be exactly opposite. A possible explanation may be that the charge characteristics of the enzyme are altered by the buffer system being used. Additional research will have to be carried out to resolve this problem.

Low immunological cross-reactivity, estimated by double diffusion on agar techniques, of anti-chicken liver FDPase with the FDPase of chicken breast muscle extract (Fig. 11) and pure, concentrated breast muscle FDPase (Fig. 12) indicates that chicken liver FDPase and chicken breast muscle FDPase are immunologically different. This is supported by the occurrence of spur formation, that is, the non-continuity of precipitin bands, between anti-serum——liver FDPase and anti-serum——breast muscle FDPase precipitin bands (Fig. 12). Spur formation is indicative of the independence of the anti-body——antigen systems being studied. Quantitative precipitin tests (Fig. 13) support the idea of immunological differences between the liver and muscle enzymes. In these tests,

undiluted anti-chicken liver FDPase gave a maximum of 84% inhibition of chicken breast muscle FDPase activity. In comparison, anti-chicken liver FDPase gave complete inhibition of chicken liver FDPase even when the anti-serum was diluted 1:16.

On the other hand, the FDPases of chicken liver and chicken kidney appear to be immunologically similar. The continuity of the anti-liver FDPase——kidney FDPase and anti-serum——liver FDPase precipitin bands (Fig. 11) and the complete inhibition of the activity of the liver and kidney FDPases by about the same amount of anti-serum in the quantitative precipitin tests (Fig. 13) indicate an immunological similarity between the two enzymes. Similar results were reported for the FDPase isozymes of the rabbit by Enser et al. (17). Immunological analyses on these isozymes showed that the rabbit liver and rabbit kidney FDPases were similar while the rabbit muscle FDPase was unaffected by anti-serum to rabbit liver FDPase in both Ouchterlony double diffusion analysis and precipiten tests.

The amino acid compositions of the FDPases of chicken liver and chicken breast muscle were found to vary significantly for most of the amino acids, when compared on the basis of the same molecular weight. The lysine, histidine, glutamic acid, proline, isoleucine, and leucine content of the two enzymes was found to vary significantly at the 1% probability level. The arginine, aspartic acid, methionine, tyrosine, and tryptophan content of the two

TABLE V

Amino Acid Composition of FDPase Isozymes from the Rabbit, Pig, and Chicken (moles / 135,000 g enzyme)

Amino Acid Residues	Rabbit			Pig	Chicken[a]	
	Liver[b]	Muscle[c]	Kidney[d]	Kidney[e]	Liver	Muscle
Lys	133	93	108	100	99	112
His	15	10	13	23	14	20
Arg	34	45	47	53	56	41
Asp	172	107	147	127	137	107
Thr	66	81	65	72	68	77
Ser	83	79	72	70	83	79
Glu	84	123	94	112	82	122
Pro	55	50	70	59	45	61
Gly	112	108	100	111	114	113
Ala	116	115	108	115	103	102
Cys	21	22	16	16	20	20
Val	99	108	89	84	108	94
Met	31	22	40	32	20	14
Ile	66	60	73	68	93	67
Leu	99	124	102	103	103	122
Tyr	42	66	48	43	54	50
Phe	43	33	43	40	41	41
Try	--	0	0	7	2	3
Total Residues	1271	1246	1235	1235	1242	1245

a Average values of two determinations for each isozyme.

b Data from Pontremoli et al. (67) recalculated for molecular weight of 135,000.

c Data from Fernando et al. (20) recalculated for molecular weight of 135,000.

d Data from Krulwich et al. (31) recalculated for molecular weight of 135,000.

e Data from Mendicino et al. (45) recalculated for molecular weight of 135,000.

enzymes was found to vary significantly at the 5% probability level. The remaining amino acids did not vary significantly.

The amino acid composition of FDPase isozymes from the rabbit, pig, and chicken are summarized in Table V. Even though there are obvious differences among the various FDPases in amino acid analysis, no consistent species or tissue patterns are evident. In order to obtain a more objective comparison of amino acid compositions, a diviation function was calculated:

$$D = \left[\sum (x_{1,i} - x_{2,i})^2 \right]^{\frac{1}{2}}$$

where $x_{1,i}$ represents the mole fraction of amino acid i in protein 1 and $x_{2,i}$ represents the mole fraction of the same amino acid in the protein being compared (26). The results presented in Table VI suggest that there is a difference in amino acid composition between homologous FDPases from different species and between heterologous FDPases within the same species. It is noteworthy that the divergence was larger in the latter comparisons. These results also show that some of the variability can be attributed to variations in analytical techniques.

The molecular weight of the FDPases of chicken liver and chicken breast muscle were estimated by chromatography on Sephadex G-200 and, in the case of the liver FDPase, from the sedimentation coefficient as determined by ultracentrifugation studies. Chicken liver FDPase

TABLE VI

Divergence of Amino Acid Composition of Rabbit, Pig, and Chicken FDPases[1]

I. Comparison of the same enzymes:				
Chicken liver (a)	x	chicken liver (b)	=	1.1
Chicken muscle (a)	x	chicken muscle (b)	=	1.1
		Average		1.1
II. Comparison of isozymes from the same species:				
Chicken liver (average)	x	chicken muscle (average)	=	5.4
Rabbit liver (67)	x	rabbit muscle (20)	=	7.6
Rabbit liver (67)	x	rabbit kidney (31)	=	3.6
Rabbit muscle (20)	x	rabbit kidney (31)	=	5.9
		Average		5.6
III. Comparison of homologous isozymes from different species:				
Chicken liver (average)	x	rabbit liver (67)	=	5.0
Chicken muscle (average)	x	rabbit muscle (20)	=	3.1
Rabbit kidney (31)	x	pig kidney (45)	=	3.1
		Average		3.7

1 Divergence = D x 100

exhibited a molecular weight of 134,900 ± 4,400 from chromatography on Sephadex G-200. Data from the sedimentation coefficient, determined by ultracentrifugal methods, yielded a molecular weight of 143,300 for chicken liver FDPase. In the case of chicken breast muscle FDPase, data from chromatography on Sephadex G-200 yielded a molecular weight of 152,050 ± 500 for the enzyme. In view of findings that isozymes of a given enzyme have approximately the same molecular weight (28, 39, 40, 49) and that the mammalian FDPases have molecular weights reported in the range of 127,000-139,500 (18, 31, 45, 67, 71), the molecular weight of 135,000 for liver FDPase is assumed to represent the true molecular weight of the avian FDPases. The high molecular weight found for chicken breast muscle FDPase may be due to aggregation of the enzyme to form a larger molecular species. The aggregation may be due to the lack of proper stabilizers, ionic strength, pH, temperature, time, or any possible combination of these effectors. This problem has yet to be completely resolved.

In, conclusion, FDPase has been isolated from the liver and breast muscle of chicken. A comparison of the molecular properties of these enzymes indicates that, as has been reported for the rabbit FDPase isozymes (17, 20), there are two distinct forms of the enzyme. One of these forms is found in the liver and kidney tissues of the chicken while the other is found in chicken breast muscle. Further work remains to determine the precise kinetic properties and physiological roles of these isozymes in the chicken.

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