pounds that serve as inhibitors of D-α-lysine mutase (Table IX) is as yet difficult to assess.

All of the inhibitors tested, with the possible exception of *n*-caproic acid and D-proline show saturation type curves similar to those of Figure 11 in plots of per cent inhibition *vs.* inhibitor concentration. Certain diamino acids closely related in structure to lysine are among the more powerful inhibitors. *S*-aminoethylcysteine is a good inhibitor of the mutase and inhibits by reason of being a nonmetabolizable substrate analog. At present there appears to be little correlation between structure and effectiveness of the inhibitors tested.

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Biological and Immunological Activity of Fructose 1,6-Diphosphatase. Application of a Quantitative Displacement Radioimmunoassay*

Helmut J. Kolb and Gerold M. Grodsky†

ABSTRACT: A displacement radioimmunologic assay of fructose 1,6-diphosphatase (FDPase) from rabbit liver was developed. Enzyme was purified and a high titer anti-FDPase serum was obtained from guinea pigs. The catalytic activity of FDPase was partially but not completely inhibited by the antiserum. Of the iodination methods tested, radioiodinated enzyme with high specific activity was best obtained by the chloramine-T procedure. Since the enzyme proved sensitive to exposure to the oxidant, an incubation time of 7–10 sec was chosen for the iodination. A constant amount of labeled FDPase was incubated in presence of varying standard amounts of unlabeled enzyme with antiserum, and preferential sodium sulfate precipitation was used for separating the bound and free FDPase. The displacement radioimmunoassay was sensitive to an enzyme concentration of 0.08 μg or 0.01 enzyme unit. The substrate and AMP did not quantitatively interfere with the assay up to concentrations of 5×10^{-3} M. During various steps in the purification of FDPase, the biologic activity per unit immunologic activity remained constant. Native enzyme was estimated quantitatively in crude 100,000*g* supernatants of tissue homogenate. The ratio of biologic to immunologic activity was the same in supernatants of rabbit liver and kidney. Supernatants of rabbit muscle, brain, heart, and spleen were immunologically inactive. Hepatic supernatants of the rat, sheep, cow, and pig did not cross-react, despite demonstrable biologic activity. Studies indicate the quantitative displacement radioimmunoassay in conjunction with biologic assays is useful to evaluate the level and structural characteristics of an enzyme in a biologic environment.

Displacement radioimmunological assays have been derived and are routinely used for the measurement of various peptide hormones including insulin (Yalow and Berson, 1960; Grodsky and Forsham, 1960), ACTH[1] (Bersen and Yalow, 1968a), glucagon (Unger *et al.*, 1959; Nonaka and Foa, 1969), growth hormone (Utiger *et al.*, 1962), vasopressin (Millrod and

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1964), are: ACTH, adrenocorticotropic hormone; TSH, thyroid-stimulating hormone; FSH, follicle-stimulating hormone; FDPase, fructose 1,6-diphosphatase.

Moses, 1969), TSH[1] (Odell *et al.*, 1965), FSH[1] (Odell *et al.*, 1968), and parathyroid hormone (Berson and Yalow, 1968b). Recently, steroid hormones coupled with albumin were determined by this technique (Abraham, 1969). The displacement radioimmunoassay is based on the decreased binding of a radioiodinated antigen to a titered amount of antibody, caused by increasing quantities of unlabeled antigen, added as standard or unknown. The free and antibody-bound antigen are separated by various methods, *e.g.*, double antibody precipitation (Hales and Randle, 1963), charcoal adsorption (Herbert *et al.*, 1965), alcohol precipitation (Heding, 1966), or salt precipitation (Grodsky and Forsham, 1960; Karam *et al.*, 1963). The per cent of radioiodine found in the bound or free fraction is a function of the antigen concentration.

In general, the displacement radioimmunoassay has proven to be a rapid and highly specific method, permitting determinations of peptide hormones in their biologic environment without further purification or concentration. The method however has not yet been applied to the measurement of enzymes and other proteins, consisting of subunits and a larger molecular weight. In conjunction with assays of biological activity, the displacement immunoassay should facilitate evaluation of synthesis and turnover of various enzymes in their biological environment.

Fructose 1,6-diphosphatase from rabbit liver, a protein with a molecular weight of 130,000 and which consists, according to Sia *et al.* (1969), of four regulatable subunits, was considered a suitable enzyme for radioimmunological studies. The following paper describes the displacement radioimmunological determination of FDPase from various tissues.

Materials and Methods

Fructose 1,6-diphosphate (tetrasodium), triphosphopyridine nucleotide, phosphohexose isomerase, and glucose 6-phosphate dehydrogenase were purchased from Sigma.

Carrier-free iodine 125 and 131 isotopes in small plastic vials were obtained from Iso-Serve Division of Cambridge Nuclear Corp.; chloramine-T was purchased from Eastman Organic Chemicals and sodium metabisulfite, potassium iodate, and potassium iodine from Allied Chemicals. Complete and incomplete adjuvant and special Agar Noble used for immunodiffusion and immunophoresis were purchased from Difco Laboratories.

Fructose 1,6-diphosphatase was isolated from rabbit livers as described (Pontremoli *et al.*, 1965). From 600 g of frozen rabbit livers, purchased from Pel-Freez, Rogers, about 6.8 mg of enzyme was obtained. The final specific activity was 120–130 units/mg of protein. The enzyme was crystallized and checked for impurities on polyacrylamide gel electrophoresis as described by Davis (1964). At pH 8.3, 7% acrylamide concentration, a single protein band was obtained after staining with Amido Black. In the analytical ultracentrifuge, using schlieren optics, the enzyme moved in a single boundary.

The biological activity of FDPase was determined with fructose 1, 6-diphosphate as substrate in 0.1 M glycine buffer (pH 9.4) as described by Pontremoli *et al.* (1965). A Gilford spectrophotometer with automatic recorder was used to follow the reaction at 340 mμ. The temperature of the reaction was controlled with a Haake thermostat with cooling unit and was kept constant at 22°.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. For the purified enzyme the protein was determined using the molecular extinction coefficient as reported elsewhere (Pontremoli *et al.*, 1965).

For the preparation of crude tissue supernatant about 1 g of wet tissue and 2 ml of 0.02 M phosphate buffer (pH 8.0) were homogenized and centrifuged at 100,000*g*.

High specific activity iodination with chloramine-T as an oxidant was modified from the standard procedure described by Hunter and Greenwood (1962). A small conical centrifuge tube with 5-ml volume was used for the iodination. FDPase (20–50 μg), fructose 1,6-diphosphate (5×10^{-4} M), and radioactive iodine (5 ml) (1 μl = 0.5 mCi) were mixed in 0.5 M phosphate buffer (pH 7.6). The iodination was initiated by the addition of 25 μl of freshly prepared chloramine-T solution (usually 4.2 mg/ml in phosphate buffer) and terminated after 7–10-sec reaction time with 100 μl of freshly prepared sodium metabisulfite solution (5 mg/ml). A Hamilton syringe was used for the rapid addition of chloramine-T and sodium metabisulfite.

The unreacted iodide was separated from the enzyme by passing the mixture over a small Sephadex column (3 g of Sephadex G-25), equilibrated with 0.05 M phosphate buffer (pH 7.6) and coated with albumin (Hunter and Greenwood, 1962). The yield of iodination in per cent was calculated from the ratio of radioactivity in the protein fraction to the total radioactivity in the sample. The iodinated FDPase was stored in an albumin solution (10 mg/ml) and kept frozen at −30°.

Radioactivity was determined by using a Model 1085 automatic gamma well counter or a table monitor, both from Nuclear-Chicago.

Anti-fructose 1,6-diphosphatase serum from guinea pigs was obtained by injecting five animals (about 300-g weight) into the toepads with 1 mg of FDPase (specific activity 130 U/mg of protein), in 100 μl of 0.02 M phosphate buffer, suspended in 100 μl of Freund's complete adjuvant at pH 7.6. In four weekly intervals the animals were injected with the same dose in incomplete adjuvant. One week after the four injections, the animals were bled by cardiac puncture. About 5 ml of antiserum was obtained from each animal. The antiserum was stored in small portions at −30°.

Double immunodiffusion of FDPase was carried out as described by Ouchterlony (1953). The agar gel concentration was 2% in 0.075 M Veronal buffer (pH 8.6). After formation of the precipitant line, the agar plate was soaked with 0.1 M NaCl solution for 2 days and with distilled H_2O for an additional day. The agar was dried using filter paper and stained for protein using a 1% solution of carmine B.

Immunophoresis was performed according to standard procedures using 2% agar gel in 0.075 M Veronal buffer (pH 8.6). Electrophoresis was run for 2.5 hr at 36 mV at 4°. Afterward, antiserum was added to adjacent large wells and the plate was kept at room temperature for 2 days. After the precipitant lines had formed, the plate was washed and stained as described for immunodiffusion.

Sodium sulfate solution (25%) was prepared by dissolving 125 g of sodium sulfate in about 350 ml of H_2O. The solution was heated to 60–65° until the sodium sulfate dissolved; the solution was added to a volumetric flask, and adjusted to 500 ml, and stored in a 25° water bath.

For the quantitative displacement radioimmunological determination of FDPase, the known standard or the sample to be analyzed was incubated in a test tube in 0.2 M glycine

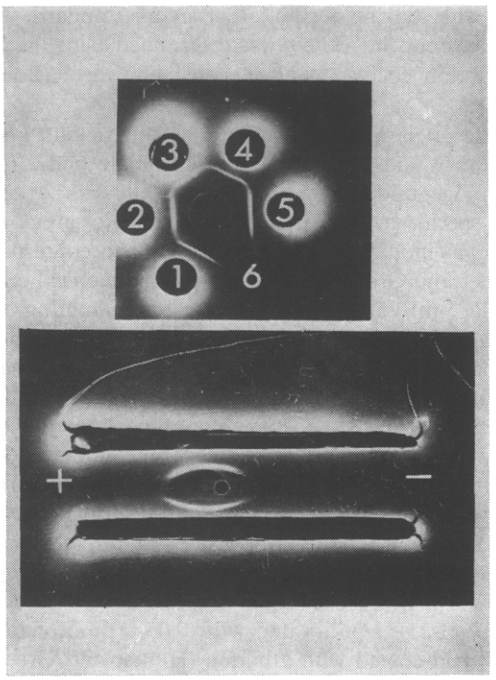

FIGURE 1: Top: double immunodiffusion of rabbit liver FDPase against antiserum from guinea pigs. The center well contained 100 μg of FDPase. Wells 1–5 contained antiserum from guinea pigs injected with enzyme, well 6 contained antiserum from a normal, untreated guinea pig. Bottom: high-voltage immunophoresis of FDPase. The center well contained 100 μg of FDPase, the troughs on each side were filled with guinea pig antiserum. For details, see text.

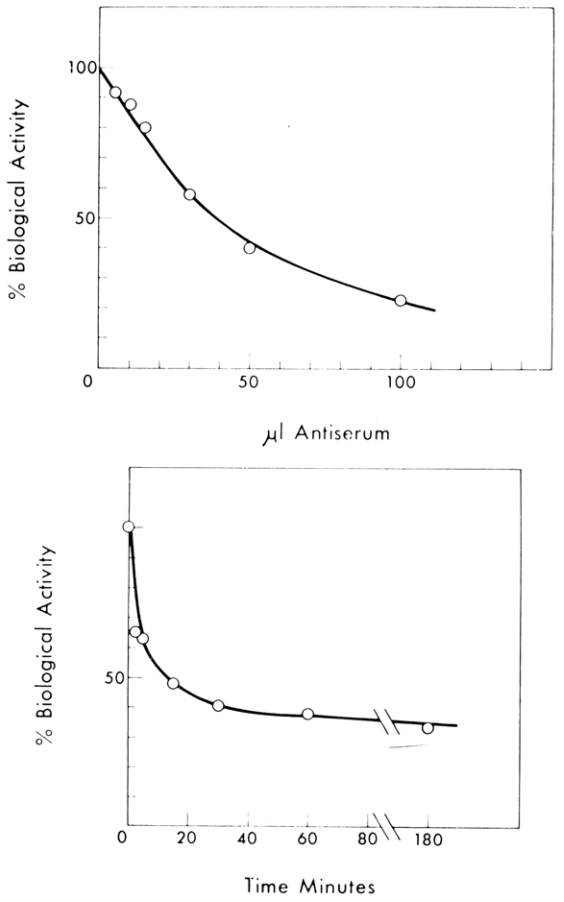

FIGURE 2: Top: inhibition of enzymatic activity of FDPase by increasing amounts of guinea pig antiserum. To 1.35 ml of 2 M glycine buffer (pH 7.6) containing FDPase, manganese, and the auxiliary enzymes necessary for determination of biological activity, antiserum and glycine buffer were added to give a total volume of 1.45 ml. After 5-min incubation with the antiserum, 50 μl of fructose 1,6-disphosphate (12 mg/ml) was added to start the enzymatic reaction, and the reaction was followed using a Gilford instrument as described under Methods. The temperature was 22°. Bottom: time dependence of antiserum inhibition. Antiserum (50 μl) was added to 1.40 ml of 0.2 M glycine buffer as described under Figure 2 (top). After incubation for the desired time, 50 μl of substrate was added and the activity was determined as before.

buffer (pH 7.6) containing 1% albumin. Antiserum, diluted to the desired concentration with 1% albumin (pH 7.6), was added and the tubes were incubated at 4°. The total volume of the incubated solution was 0.6 ml.

After the incubation period (usually 16 hr), 0.3 ml of a 1% solution of albumin (pH 7.6), containing bovine γ-globulin (5 mg/ml) as carrier protein, and radioiodinated FDPase (about 0.001–0.01 mμg of FDPase) were added to the test tubes. A similar aliquot was added to two separate counting tubes for determination of total counts. After incubation at 25° for 25 min, 2.2 ml of 25% sodium sulfate solution was added; the tubes were shaken carefully and kept at 25° for an additional 30 min. The tubes were then centrifuged for about 10 min at 3000 rpm at room temperature and an aliquot of the supernatant was counted. Each sample was assayed in duplicate or triplicate.

Results

Double Immunodiffusion and Immunophoresis of FDPase. The presence of antibody against rabbit liver FDPase in serum obtained from guinea pigs immunized with the enzyme is demonstrated by double immunodiffusion. Figure 1 (top) shows the single precipitant lines formed by antiserum (wells 1–5). No precipitant line appeared during the identical period near well 6, containing normal guinea pig serum. Patterns after immunophoresis are shown in Figure 1 (bottom). Only a single precipitant line was formed after electrophoresis of the enzyme and subsequent double diffusion.

Inhibition of FDPase Activity by Antiserum. When fructose 1,6-diphosphatase in 0.2 M glycine buffer (pH 7.6) was treated with increasing amounts of antiserum, the catalytic activity of the enzyme was inhibited (Figure 2, top). As shown in Figure 2 (bottom), about 40–60% of the biologic activity was inhibited after 5-min preincubation with 50 μl of antiserum. After 25-min preincubation, a maximum of 70% inhibition was approached. The same order of inhibition of FDPase activity was also observed in supernatants of rabbit liver and kidney.

High Specific Activity Iodination of FDPase. To achieve high sensitivity of a radioimmunoassay in the millimicrogram range, tracer protein with high specific activity is required and is usually obtained by iodination with ^{125}I or ^{131}I isotopes. The radioiodination procedure using ICl as described by Izzo *et al.* (1964) resulted in specific activities too low to be useful.

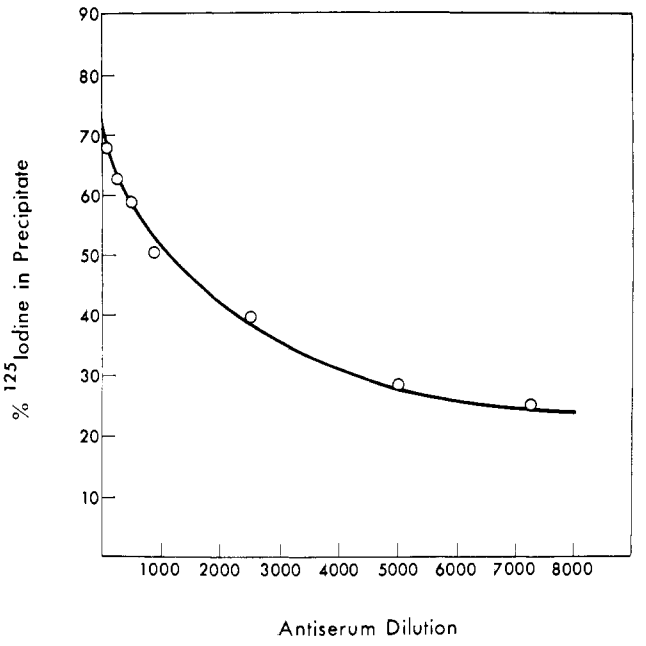

FIGURE 3: Immunodisplacement of iodinated FDPase at different concentration of antiserum; 0.6 ml of 0.2 M glycine buffer (pH 7.6) containing 1% albumin and 0.005 mμg of radioiodinated FDPase and different dilutions of guinea pig antiserum was incubated for 16 hr at 4°. Bound enzyme was precipitated with Na_2SO_4 as described in text.

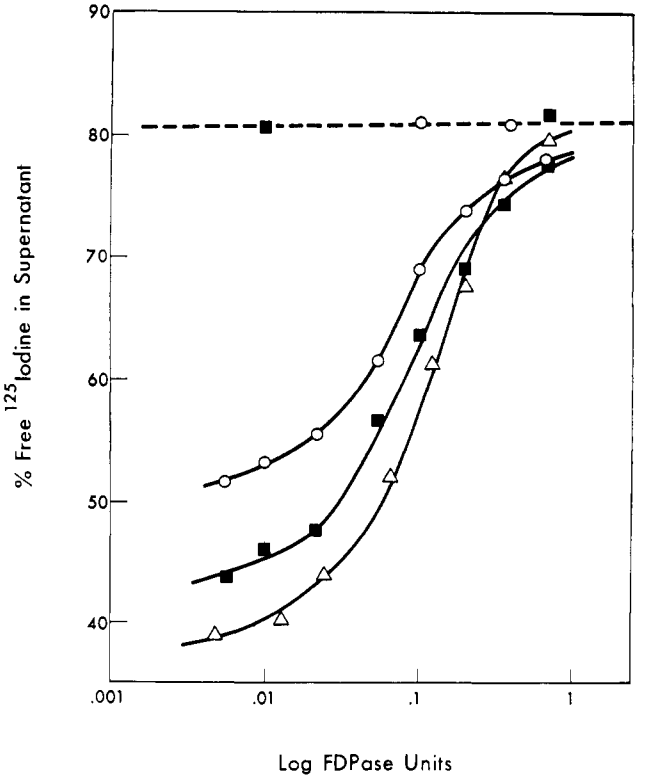

FIGURE 4: Effect of incubation time on standard curves for the quantitative determination of FDPase. (○—○) 1 hr, (■—■) 8 hr, and (△—△) 16 hr. Dashed line shows control studies performed in the absence of antibody.

The radioiodination procedure using chloramine-T as an oxidant for radioiodide as described by Hunter and Greenwood (1962) was previously used for the high specific activity iodination of insulin and other hormones. Table I shows the influence of the incubation time with chloramine-T on the yield of iodinated FDPase. Efficiency of iodination increased with incubation time and increased concentration of chloramine-T. In contrast to most peptide hormones, FDPase proved to be sensitive to exposure with chloramine-T under conditions used for the iodination. Even when the incubation time was reduced to 60 sec, 95% of the catalytic activity of the enzyme was lost. Furthermore, the enzyme showed a decreased solubility in Na_2SO_4 solution, which caused a flattening of the standard curve and a decrease in the sensitivity of the assay. There was only a small decrease in efficiency of iodination when the incubation time with chloramine-T was decreased from 60–7 sec (Table I). However, the solubility and biological activity of the labeled FDPase more closely approximated that of native enzyme and a sensitive standard curve was obtained. Consequently, for all experiments described in this paper an incubation time of 7–10 sec was chosen.

Influence of Antiserum Concentration on Binding of [^{125}I] FDPase. Binding of [^{125}I]FDPase as a function of antiserum concentration was determined by incubating radioiodinated FDPase with different dilutions of antiserum. The percentage of ^{125}I precipitated dropped in a curvilinear fashion with dilution of the antiserum (Figure 3). Similar curves were obtained using antisera from the other four injected animals. For the immunoassay, an antiserum dilution of 1:5000 was used which bound about 30% of the labeled enzyme.

Standard Curve for the Quantitative Determination of FDPase. When the per cent of iodine isotope found in the supernatant was plotted against the logarithm of added unlabeled FDPase, standard curves were obtained. As Figure 4 demonstrates, the curves possess a linear portion, where the amount of radioiodinated FDPase is proportional to the logarithm of the amount of FDPase added to the incubation tube. This area provided a working range of about 0.01–0.5 enzyme unit, or, assuming a specific activity of 130 units/mg of protein, 0.08–4.0 μg of enzyme.

The influence of time of the initial incubation with antibody

TABLE I: Iodination with ^{131}I of Fructose 1,6-Diphosphatase Using Chloramine-T.

Amt of Enzyme (μg)	Moles of Chloramine-T Used	Incubation Time (sec)	Yield of Iodination (%)	Sp Act. (mCi/mg of Protein)	Biol Act. (%)
30	3×10^{-8}	60	0	0	
40	10^{-7}	60	5	3	32
40	10^{-7}	300	8	5	3
26	3×10^{-7}	7	28	26	65
26	3×10^{-7}	20	26	25	35
26	3×10^{-7}	60	34	32	5
20	3×10^{-7}	300	69	87	0

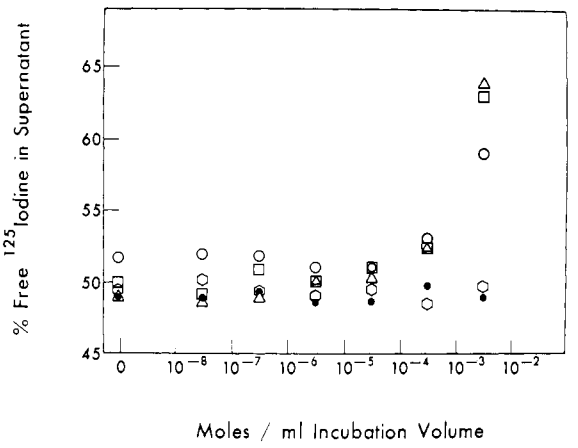

FIGURE 5: Influence of small molecular compounds on the immunodisplacement of iodinated FDPase. Conditions were identical with those of the "zero" point of a typical standard curve, but included varying concentrations of the following: (△) AMP, (□) ATP, (○) fructose 1,6-diphosphate, (○) $MnCl_2$, and (●) NaCl.

TABLE II: Comparison between Biological and Radioimmunological Determination of FDPase from Rabbit Liver Following the Purification Procedure as Described by Pontremoli *et al.* (1965).

	Bioassay (Units/ml)	Immunoassay (Units/ml)[a]
Soluble liver supernatant (100,000*g*)	12.4	11 ± 1.6
Lactic acid fraction (pH 4.0)	10.8	10 ± 1.5
Ammonium sulfate precipitate (55–62%)	33.6	30 ± 2.5
Heat-stable fraction (50°)	30.8	31 ± 2.5

[a] ± = std error.

on the sensitivity of the displacement immunoassay was also investigated (Figure 4). Sensitivity of the standard curve increased with increasing incubation time up to 16 hr; in additional experiments, incubation for 24–48 hr had little further effect. Thus, in most experiments an incubation of overnight (16 hr) was chosen, though good precision in the assay was obtained even after 1 hr.

The influence of pH on the displacement immunoassay was also investigated. When the assay was performed between pH 4 and 10 under standard conditions, maximal binding to antibody occurred between pH 7.0 and 8.6; at pH values under 6, dissociation of the antigen–antibody complex resulted in a flat standard curve. For all other assays, a pH of 7.6 was chosen.

Trasylol, a proteolytic enzyme inhibitor, which has been used previously for the radioimmunoassay of glucagon (Hazzard *et al.*, 1968) was without effect on the immunoassay when added in concentrations up to 500 units/tube and was deleted in subsequent experiments.

Influence of Fructose 1,6-Diphosphate, Adenosine Monophosphate, and Manganese on the Immunodisplacement of FDPase. Pontremoli *et al.* (1968) demonstrated the binding of the substrate fructose 1,6-diphosphate to FDPase in absence of manganese ions. Also adenosine monophosphate, an allosteric inhibitor of the enzyme, tightly binds with the enzyme (Pontremoli *et al.*, 1966). Since the assay is used for the estimation of the enzyme in cytosol, the influence of increasing concentrations of FDP, AMP, ATP, $MnCl_2$, and NaCl on immunodisplacement was examined. The results of this study are shown in Figure 5. There was no interference by NaCl or $MnCl_2$ up to 5×10^{-2} M concentrations. Fructose 1,6-diphosphate, AMP, and ATP interfered at concentration higher than 5×10^{-3} M; however, this amount is several orders of magnitude over normal tissue levels.

Radioimmunodetermination of FDPase from Rabbit Liver at Different Stages of Purification. The displacement radioimmunoassay was used to estimate FDPase levels in crude fractions during purification of the enzyme. Table II compares the enzyme units of FDPase as obtained by the biological and radioimmunological assay, following four steps of the purification procedure as described by Pontremoli *et al.* (1965). The results of these two assays, though based on completely different principles, were identical within the limits of error.

Radioimmunodetermination of FDPase in 100,000g Supernatants of Various Tissues. Since the protein content of crude 100,000*g* supernatants is high (varying from about 50 mg/ml for liver to about 15 mg/ml for muscle), the effect of increasing amounts of supernatant on the nonspecific coprecipitation of [^{125}I]FDPase in absence of antiserum was investigated. As seen in Figure 6 about 20% of the radioiodinated FDPase was coprecipitated nonspecifically in the absence of antiserum or any supernatant. Up to 20 μl of 100,000*g* supernatant of liver could be added without causing additional precipitation; when more hepatic supernatant was added, nonspecific coprecipitation increased, thereby reducing the specificity and sensitivity of the assay. An excess of supernatant resulted in heavy, dense precipitates instead of the fine precipitate formed when pure enzyme standards or when small volumes of hepatic supernatant were used. As also seen in Figure 6 up to 90 μl of 100,000*g* supernatant from rabbit muscle could be added without additional coprecipitation of radioiodinated FDPase. For the immunological determination of FDPase in supernatants from various tissues, only that amount was used which did not cause additional coprecipitation over the controls.

In Figure 7 the proportional cross-reaction of enzyme in 100,000*g* supernatants from various rabbit tissues was compared to standard samples of the purified hepatic enzyme. The supernatants of liver and kidney cross-reacted identically and proportionally at different dilutions along the standard curve, indicating a high degree of chemical identity with the purified enzyme; muscle supernatant, however, did not cross-react. The result is in good agreement with the finding of Enser *et al.* (1969), who observed that rabbit muscle FDPase was not inhibited by goat anti-rabbit liver FDPase serum.

As shown in Table III the small amounts of biological activity in rabbit brain, heart, spleen, and muscle were paralleled by the failure to detect immunologically cross-reacting

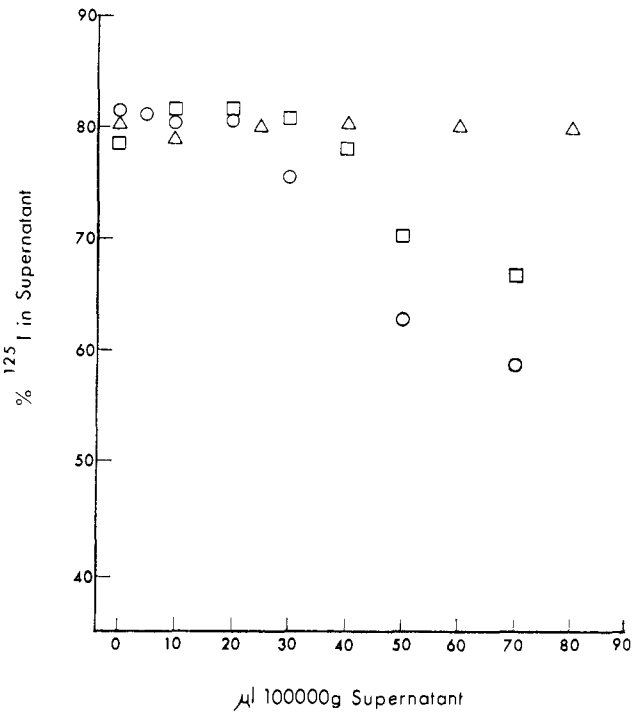

FIGURE 6: Effect of increasing amounts of tissue 100,000*g* supernates on the nonspecific coprecipitation of [^{125}I]FDPase. Increasing amounts of tissue 100,000*g* supernatants were incubated under conditions used for the assay with a constant amount of [^{125}I]FDPase, but in the absence of antiserum. (○) Rabbit liver supernatant, (□) rabbit kidney supernatant, and (△) rabbit muscle supernatant.

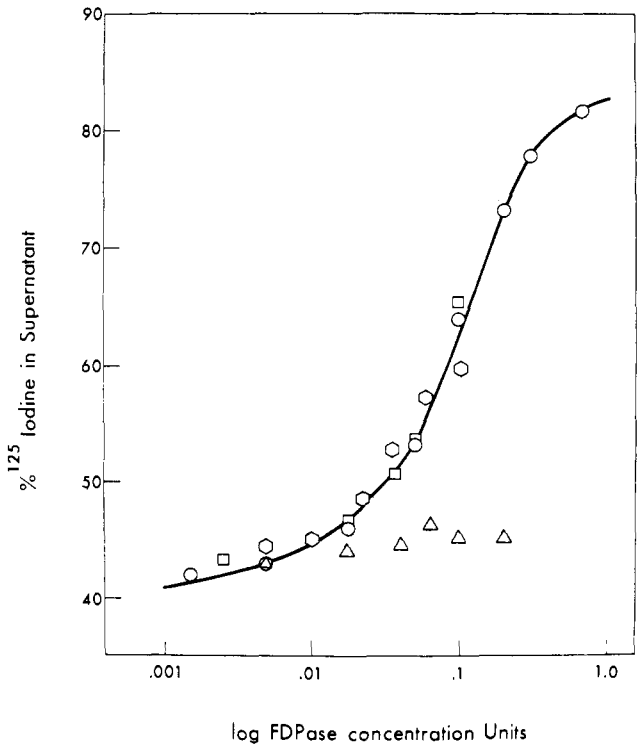

FIGURE 7: Comparison of proportional displacement of FDPase in different rabbit tissues. The radioimmunoassay was carried out as described under Methods. (○—○) Rabbit liver enzyme, purified, specific activity 130 U/mg protein; (□—□) 100,000*g* supernatant of rabbit liver; (⬡—⬡) 100,000*g* supernatant of rabbit kidney; (△—△) 100,000*g* supernatant of rabbit muscle.

material in these tissues. The kidney enzyme, in contrast, was in significant quantity and had the same ratio of biologic to immunologic activity as the hepatic enzyme.

Hepatic supernatants from different species were also investigated (Table III); different amounts of supernatants of rat, pork, beef, and sheep liver were assayed under standard conditions for FDPase using the displacement radioimmunoassay. None of the nonrabbit tissue cross-reacted in the displacement immunoassay despite the presence of significant levels of enzymatic activity. Thus, the FDPases in the investigated species seem to be different in their immunologic structure from the rabbit liver enzyme.

Discussion

Fructose 1,6-diphosphatase from rabbit liver was considered a suitable model enzyme for the application of the displacement radioimmunoassay. As our results demonstrate, FDPase can be determined quantitatively and specifically by this method which in conjunction with measurements of enzymatic activity, permits comparison of structural and functional relationships in crude biologic preparation.

The sensitivity of the current displacement radioimmunoassay was as great as that of the bioassay. However, studies of the displacement radioimmunoassay for peptide hormones indicate additional sensitivity is both theoretically and practically limited only to the availability of high-titer antisera and the specific activity of the labeled protein used as a marker (Berson and Yalow, 1968b). Previous experience with peptide hormones suggest higher titer antisera could be produced by a prolonged immunization regimen. Sensitivity as low as 1 μμg of ACTH/ml has been reported by Berson and Yalow (1968b) using this technique.

Immunologic techniques for the determination of enzymes in biologic fluids have been used (Granner *et al.*, 1968; Doyle

TABLE III: Determination of FDPase in Homogenates from Different Tissues and Species Using the Biological and Radioimmunological Assay.

Species	Tissues	Bio assay (Units/ml of 100,000*g* Supernatant)	Radioimmunoassay (Units/ml of 100,000*g* Supernatant)
Rabbit	Liver	9.6	10.1 ± 1.6
Rabbit	Kidney	6.2	5.3 ± 1.4
Rabbit	Brain	0.5	0
Rabbit	Heart	0.1	0
Rabbit	Spleen	0.1	0
Rabbit	Muscle	3.2	0
Rat	Liver	6.3	0
Beef	Liver	6.7	0
Pork	Liver	19.5	0
Sheep	Liver	9.1	0
Horse	Liver	8.4	0

and Shimke, 1969). The displacement radioimmunoassay is advantageous in that its end point of quantitative measurement is established with isotopic precision and convenience and it is not dependent on the spontaneous precipitation of complicated natural antigen–antibody complexes or upon complement. This last phenomenon often interferes in immunological assays where constant aggregate formation in crude biologic fluids is assumed (Morgan *et al.*, 1964; Samols and Bilkers, 1964). Difficulties were encountered in initial attempts to produce a high specific activity [^{125}I]FDPase, since the enzyme is labile to oxidants. Iodination of proteins to achieve high specific activity usually requires the use of carrier-free radioiodine and microgram quantities of the protein to be iodinated. Because of the low concentration of the reactants, the efficiency of iodination is affected by contaminations, side reactions, and decreased reaction rates. The ICl method, previously used by Izzo *et al.* (1964), gave low yields. By applying the chloramine-T technique of Hunter and Greenwood (1962) and reducing the exposure time to 7–10 sec, [^{125}I]FDPase with adequate specific activity and sufficient immunological activity to provide sensitive standard curves was obtained. Fortunately, in the displacement radioimmunoassay the labeled protein marker need not be identical in immunological or biological activity with the unlabeled protein being measured (Karam *et al.*, 1963; Berson and Yalow, 1968c). The reliability of the assay is based solely on the comparative effects of unlabeled standards and unknowns to displace labeled material from antibody.

It is not clear why our antiserum could inactivate only about 70% of the biologic activity of the purified enzyme, as well as FDPase activity in rabbit liver and kidney supernatant. Since more than one population of antibodies are known to be produced during immunization (Berson and Yalow, 1959), it may be that some of the heterogeneous antibodies blocked the biologically active site while others did not. It is also possible, that the enzyme–antibody complex represents a new "modified" enzyme whose kinetic properties are different from the native enzyme.

The specificity of the assay was indicated by the fact that 100,000g supernatants of hepatic tissue from other species neither increased nor decreased displacement, but gave readings of zero. Though fructose 1,6-diphosphate, AMP, and manganese are known to bind to FDPase, and influence its biological activity, concentrations of these agents in the physiologic range did not influence the immunoassay. Thus measurements of the molar concentration of enzyme can be made in cytosol preparations under conditions in which biological assays might be affected.

The identical proportional and quantitative cross-reaction of rabbit kidney and liver enzyme with the purified crystalline enzyme standard, indicates FDPase from these two organs is comparatively similar in structure, an observation also suggested by studies of Enser *et al.* (1969). It is emphasized that failure to note immunological differences does not prove identical structure, but only indicates that antigenic sites which bind a particular antiserum were unaffected. Possibly other antisera prepared in different species or strains of animals could detect such structural differences if they exist (Hayashida, 1969). In contrast, our demonstration of no cross-reaction of endogenous FDPase in liver of pig, cow, sheep, and rat, despite the high amounts of detectable biologic FDPase activity, is strong evidence that the rabbit liver and kidney enzyme differ in structure from each of these.

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