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CONCENTRATION OF LIVER AND KIDNEY FRUCTOSE-1,6-DIPHOSPHATASE DETERMINED BY SPECIFIC RADIOIMMUNOASSAY

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Summary

A radioimmunoassay for liver fructose-1,6-diphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) has been developed based on maintenance of its tetrameric structure and immunologic integrity after iodination by the Bolton-Hunter technique. The assay detected as little as 2 ng of standard enzyme. Nonspecific interference by tissue components did not occur. Enzyme concentration (μmol/1000 g tissue wet weight) was measured in tissue extracts of 49 rabbits subjected to a variety of conditions. In animals fed a 'balanced' diet containing 50—60% carbohydrate (by weight), the concentration in liver was 3.4 μM ± 0.3. After fasts of 48, 72, or 96 h, the concentration in liver increased approximately 1.4-fold. A high-fat diet did not alter the concentration significantly but a high-protein diet caused an increase of 2.1-fold to 7.2 μM ± 1.4. The greatest concentrations, 8.7 μM ± 1.9, were observed in the livers of severely diabetic rabbits. The increase paralleled the increasing severity of diabetes and provides one explanation for the augmented gluconeogenesis which occurs in the diabetic state. Changes were less marked in kidney. The greatest apparent increase, from 2.6 μM ± 1.1 in the normal fed rabbit to 4.7 μM ± 2.8, occurred in the severely diabetic animal. However, variation was sufficiently great in kidney to render apparent increases during fasting, protein feeding and diabetes statistically insignificant. For the most part changes in assayable activity followed changes in enzyme concentration except in the rabbits maintained on high-protein diets. In these, liver enzyme concentration increased by 2.4-fold whereas activity increased by only 1.3-fold, and the kidney enzyme concentration increased 1.3-fold whereas activity decreased by 20%.

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Introduction

To what extent are the intracellular reactions of regulatory enzymes controlled by changes in concentration rather than by allosteric or covalent modification of the enzymes? Since maximum-velocity assayable activity measurements of crude tissue extracts cannot distinguish between changes in concentration and catalytic state, we have developed radioimmunoassays for numerous regulatory enzymes of complex quaternary structure. In this paper we report on the chemical concentration (mol/1000 g wet wt tissue) of fructose-1,6-diphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) in liver and kidney of rabbits subjected to different nutritional, metabolic and hormonal conditions. The concentration data obtained by radioimmunoassay are compared with those obtained by activity measurements. Specific activity based upon units of enzyme activity/nmol enzyme can now be calculated.

Methods and Results

Enzyme purification and assays

Rabbit liver fructose-1,6-diphosphatase was obtained from the Sigma Chemical Co., St. Louis, MO. Their assay, at pH 9.5 gave 5 U/mg at 25°C. Rabbit muscle fructose-1,6-diphosphatase was purchased from Boehringer Mannheim Biochemicals of Indianapolis, IN, whose assay disclosed 4 U/mg at 25°C. We submitted both to further purification by preparative polyacrylamide gel electrophoresis. A preparative polyacrylamide gel electrophoretic apparatus of original design was employed. The details of the method can be found elsewhere [1]. By this procedure a 3—4-fold purification was achieved with 50—60% recovery of the liver enzyme protein. When subjected to analytical polyacrylamide anionic disc gel electrophoresis [2] the protein in the peak fractions migrated as a single protein band. When incubated at 50°C for 10 min in 8 M urea, 1% (w/v) dodecyl sulfate and 1% β-mercaptoethanol and then electrophoresed, two major protein bands were evident (Fig. 1) which is consistent with published reports that the native subunits of M_r 36 000 can undergo transformation to 29 000 by proteolytic modification [3—5]. Thus, this enzyme preparation apparently contains both subunit types. Minor slower moving bands were evident when the gels were greatly overloaded as in Fig. 1. These proteins could not be eliminated by even a combination of Sephadex® G-150 gel filtration, preparative gel electrophoresis and substrate elution from CM-cellulose [6].

Enzyme solutions were assayed for fructose-1,6-diphosphatase activity spectrophotometrically at 340 nm in a Gilford 240 spectrophotometer attached to a Gildford 6040 recorder by modification of a previously described assay [7]. Assays were carried out in a total volume of 2.0 ml at 25°C with the final concentrations for assay of the purified muscle enzymes as follows: 100 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 0.1 mM disodium EDTA, 0.32 mM sodium NADP, 7 units phosphoglucoisomerase and 0.7 unit glucose-6-phosphate dehydrogenase. Fructose 1,6-diphosphate (0.15 mM) was added to start the reaction. For assay of the purified liver enzyme and of all tissue extracts

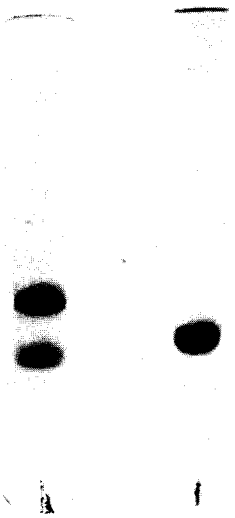

Fig. 1. Analytical dodecyl sulfate polyacrylamide disc gel electrophoresis. Purified fructose-1,6-diphosphatase was incubated for 1 h at 25°C in a solution of 8 M urea, 1% (w/v) dodecyl sulfate and 0.1 mM dithiothreitol. Approximately 25 μg protein was electrophoresed on polyacrylamide gels containing dodecyl sulfate from top to bottom until tracking dye reached the end of the gel. Liver enzyme on the left; muscle enzyme on the right.

the above assay mixture contained 40 mM $(NH_4)_2SO_4$. Black et al. [8] observed a marked stimulation of liver fructose-1,6-diphosphatase activity in the presence of $(NH_4)_2SO_4$. In assays on tissue extracts an AMP removal system was always included [8].

Antibodies to purified enzymes in this and the following paper were raised in goats. When a high titer was obtained the γ-globulin was precipitated from the antiserum with 16% Na_2SO_4 (w/v), dissolved in 0.15 M NaCl, dialyzed against water, lyophilized and stored at −40°C. Antibody against goat γ-globulin was raised in a donkey and used as second-stage antibody in the radioimmunoassays. Antibody preparations were evaluated by agar double diffusion in terms of recognition and specificity for the commercial enzyme preparations, the enzymes after purification, and the enzymes in the supernant fractions of 20% homogenates of rabbit skeletal muscle, heart, liver, and kidney. Double-diffusion studies were carried out at 25°C in 1.0% agar gels containing 1.0% NaCl and 0.1% NaN_3 (4 ml agar/7.5 X 2.5 cm glass slide). Antibody raised to the rabbit liver enzyme reacted to preparations of both the purified enzyme and liver extract with a single continuous precipitin line. The antibody also gave a continuous band with the purified enzyme and kidney extract. This agrees with other evidence [9] that the liver and kidney enzymes are immunochemically related even though they may differ in amino acid composition and structure [10,11]. The antibody gave a precipitin band with skeletal muscle extract; however, this band was not continuous with that to purified enzyme. Antibody raised to the rabbit muscle enzyme reacted with extracts of heart and muscle

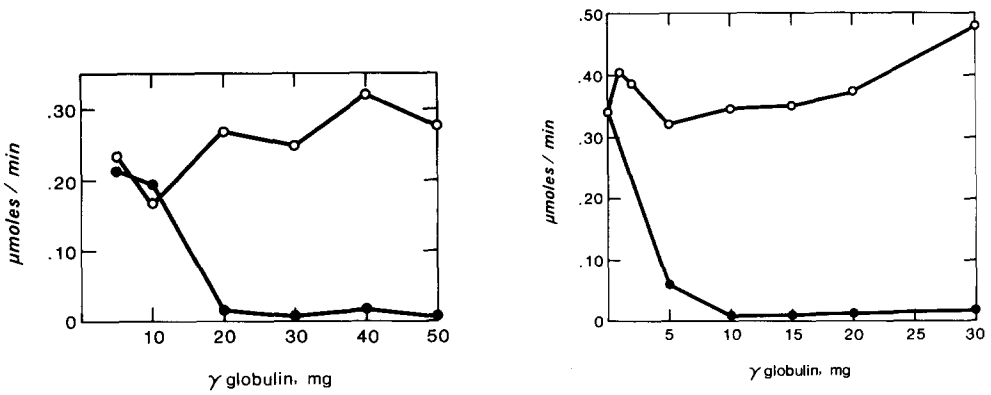

Fig. 2. Evaluation of reactions between liver and muscle enzymes and specific antibodies: Assayable enzymic activity remaining in supernatants. ○, non-immune γ-globulin; ●, specific antibody. (A) Liver fructose-1,6-diphosphatase. (B) Muscle fructose-1,6-diphosphatase.

with a single band, but formed a precipitin line with extracts of liver and kidney which was not continuous with that to heart and muscle.

For the liver enzyme inhibition studies by specific antibodies, 120 μg of commercially obtained liver enzyme was incubated with anti-enzyme γ-globulin solutions containing 5—50 mg protein, 100 μl of 0.4 M Tris-HCl buffer (pH 7.6) and enough 0.154 M NaCl to make a final volume of 610 μl. For the muscle enzyme, 100 μg of commercially obtained fructose-1,6-diphosphatase was incubated with anti-enzyme γ-globulin solutions containing 1—30 mg protein, 100 μl of 0.4 M Tris-HCl buffer (pH 7.6) and enough 0.154 M NaCl to make a final volume of 410 μl. Corresponding amounts of non-immune γ-globulin were used in control experiments. Both control and experimental mixtures were allowed to stand for 24 h at 4°C. The incubation mixtures were centrifuged at 5000 × *g* for 30 min and aliquots of the supernatant fraction (100 μl for the muscle enzyme and 150 μl for the liver enzyme) were assayed. Fig. 2a and b show that between 10 and 20 mg of the anti-liver fructose-1,6-diphosphatase γ-globulin preparation inhibited the liver enzyme (120 μg) whereas 5—10 mg anti-muscle fructose-1,6-diphosphatase inhibited the muscle enzyme (100 μg). Cross-reactivity experiments showed that antibody to the muscle enzyme also inhibited the liver enzyme. However, antibody to the liver enzyme did not inhibit the muscle enzyme. The amino acid composition of the muscle enzyme is different from that of the liver and kidney enzymes [10]. There may be regions of local similarity, however, which would explain the interaction of the liver enzyme and anti-muscle enzyme antibody.

White New Zealand male rabbits were anesthetized with ether and exsanguinated from bilaterally severed carotid and jugular vessels. Portions of liver, kidney, anterior tibialis, and cardiac left ventricle were homogenized at 0°C by Polytron apparatus in 4 vols. of 0.25 M sucrose which contained 0.1 mM disodium EDTA, 1 mM dithiothreitol and 0.1 mM Tris-HCl, pH 7.6. The 27 000 × *g* supernatant fractions were incubated separately with anti-enzyme γ-globulin and handled as in the enzyme inhibition studies. Results may be found in Figs. 3a—c.

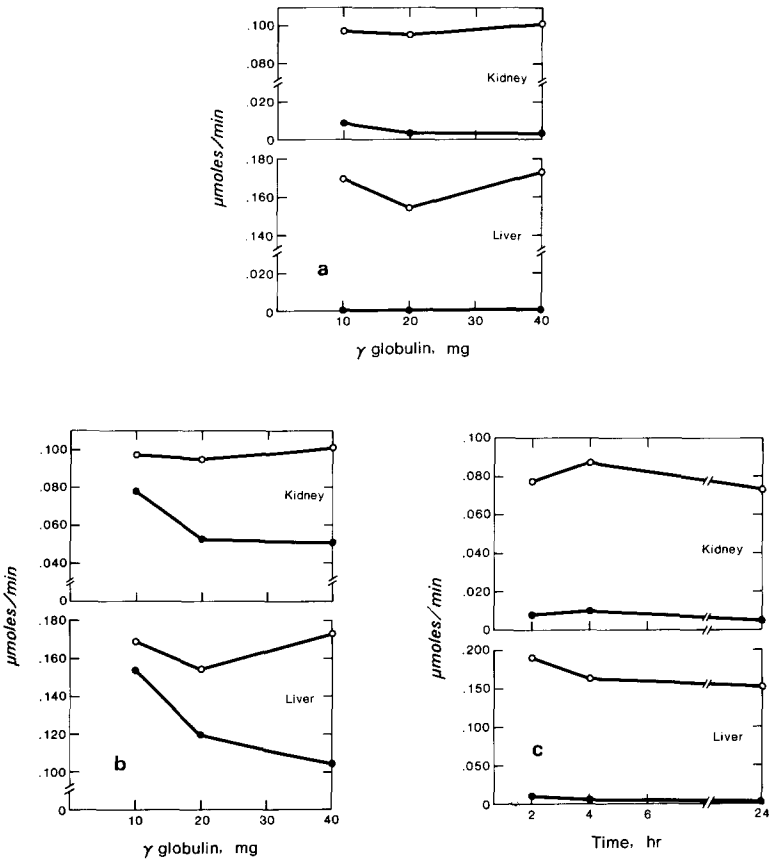

Fig. 3. Evaluation of reactions between enzymes of tissue extracts and specific antibodies (•). Assayable enzyme activity remaining in supernatants. In (a) and (c) antibody was that raised to the liver enzyme. In (b) antibody was that raised to the muscle enzyme. ○, non-immune γ-globulins.

Purified fructose-1,6-diphosphatase (15 μg) was labeled with ^{125}I according to Bolton and Hunter [12]. TAGIT® (Calbiochem. Co.) or 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester (0.8 nmol) was iodinated to a specific activity of at least 10 mCi/μg. We labeled to the extent of one ^{125}I atom/tetramer. The labeled protein and free reagent were separated on a (30 X 1 cm) column of Biogel® P-10 equilibrated with 0.05 M $Na(H)PO_4$, pH 7.5, +0.25% gelatin. The labeled protein was further purified on a (100 X 1 cm) column of Sephadex® G-150 (40—120 mesh) equilibrated in 0.05 M $Na(H)PO_4$, pH 7.5.

The purification of the labeled fructose-1,5-diphosphatase on Sephadex® G-150 is shown in Fig. 4. The major peak of radioactivity eluted at an elution volume which corresponded to that of unlabeled fructose-1,6-diphosphatase. These fractions were pooled and subsequently used for radioimmunoassays after appropriate dilution in egg albumin buffer. The pooled fraction was subjected to dodecyl sulfate polyacrylamide disc gel electrophoresis. The gels were frozen, sliced at 2.5-mm intervals and counted for radioactivity. Two

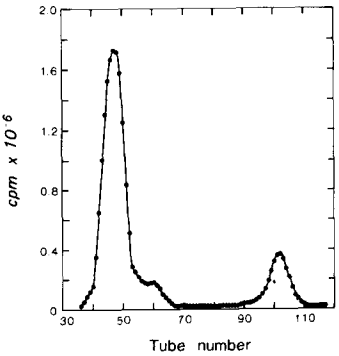

Fig. 4. Sephadex® G-150 chromatography of ^{125}I-labeled fructose-1,6-diphosphatase. 15 μg of purified liver fructose-1,6-diphosphatase, labeled by the method of Bolton and Hunter [12] was applied. 1.0 ml fractions were collected.

bands of radioactivity accounting for 96—98% of that applied were detected with R_F values similar to those of the unlabeled fructose-1,6-diphosphatase subunits. The Bolton-Hunter procedure for radioiodination apparently produced little damage to this enzyme. In contrast both the chloramine T [13] and lactoperoxidase [14] methods produced labeled enzyme peaks with elution volumes on Sephadex G-150 differing significantly from that of the unlabeled enzyme.

The interaction of each preparation of labeled fructose-1,6-diphosphatase and specific anti-fructose-1,6-diphosphatase γ-globulin was investigated by incubating approximately 6 ng of ^{125}I-labeled fructose-1,6-diphosphatase with increasing amounts of specific antibody for 48 h at 4°C in 1 ml egg albumin buffer (0.1 M sodium phosphate buffer, pH 7.5, 1% ovalbumin, 0.02% NaN_3). A second-stage antibody system, consisting of 50 μl of a 1 : 50 dilution of goat normal serum and 2 mg of donkey anti-goat IgG γ-globulin, was added and the incubation continued for 4 h in the cold. The mixtures were first counted in a γ-counter and then centrifuged at 1500 × *g* for 10 min and the supernatants aspirated. The pellets were then counted and the percentage of the original total counts in the pellet was calculated. Usually 7—10 μg of the anti-fructose-1,6-diphosphatase antibody preparation was required to produce 40—50% precipitation of ^{125}I-labeled fructose-1,6-diphosphatase.

The radioimmunoassay standard competitive binding curve was obtained by incubating a constant amount of ^{125}I-labeled enzyme (15 000 cpm) with increasing amounts of unlabeled enzyme and enough γ-globulin to produce 40—50% precipitation, as determined above, in buffer for 48 h at 4°C. Standard enzyme was quantitated by a fluorescamine method [16]. The second-stage antibody system was then added and samples treated as previously described. Background (first-stage antibody omitted) was 2—3% and subtracted from initial calculations of percent precipitated before conversion of those values into percent of control. The 100% of control value was obtained from the incubation tube which had no unlabeled enzyme added. Percent of control versus ng of unlabeled enzyme added was plotted and linearized by computer using the log logit equations of Rodbard [15]. For tissue enzyme radio-

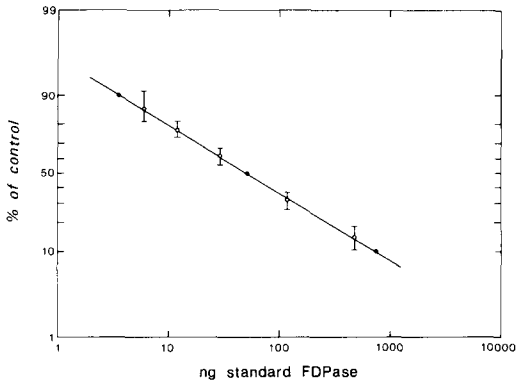

Fig. 5. Competitive binding curve. 6 ng of ^{125}I-labeled fructose-1,6-diphosphatase was incubated with 7 μg anti-fructose-1,6-diphosphatase γ-globulin and increasing amounts of unlabeled fructose-1,6-diphosphatase. ○, mean values from five sets of duplicates, the bars the S.D. ●, points determined by linear regression analysis.

immunoassay, diluted 114 000 × *g* supernatant fractions from 20% homogennates were used. Tissue assays were done in duplicate using four different dilutions.

The addition of unlabeled purified liver fructose-1,6-diphosphatase to incubation mixtures of labeled liver fructose-1,6-diphosphatase and specific antibody produced competitive binding curves such as that shown in Fig. 5. This is a composite of five different standard curves with the means plotted and S.D. represented by the bars. The effective range of these assays was from 3 to 700 ng of fructose-1,6-diphosphatase. These standard curves remained unaltered (except to detect endogenous extract enzyme) when dilute rabbit liver was added to the incubation mixtures.

To check the specificity of the antibody preparation the following rabbit enzymes of carbohydrate metabolism (5—500 ng) were added in the place of unlabeled fructose-1,6-diphosphatase; liver pyruvate kinase, muscle pyruvate kinase, muscle phosphofructokinase and muscle fructose-1,6-diphosphatase. No competition was observed at these concentrations with the labeled fructose-1,6-diphosphatase.

Fructose-1,6-diphosphatase in liver and kidney in various nutritional states and diabetes

Rabbits weighing 1500—2500 g were fed one of four diets ad libitum for at least 7 days before killing. One group was given Purina Rabbit Chow Checkers® feed. The composition by weight which was guaranteed by analysis consisted of at least 16% protein, 2% fat, 18% crude fiber, and 50—60% carbohydrate. High-carbohydrate, protein, and fat diets were prepared by Teklad Test Diets of Madison, WI. The high-carbohydrate diet by weight consisted of 61% carbohydrate, 11% protein and 5% fat. The high-protein diet, 13%, 55%, and 8%, respectively. The high-fat diet, 40%, 11% and 15%, respectively. All diets provided 3.4 kcal/g and adequate amounts of vitamins and minerals. Daily intake was not less than 80 g. Rabbits were maintained for at least one week on

rabbit chow, high-carbohydrate, high-fat or high-protein diets. Prior to killing animals were deeply anesthetized in an ether jar. Exanguination was then carried out through severed cervical vessels. Portions of liver and kidney were taken and homogenized as described for the tissue inhibition studies. Homogenates were centrifuged for 1 h at 114 000 × *g* and supernatant fractions were promptly analyzed for the concentration of fructose-1,6-diphosphatase by radioimmunoassay, for the activity of fructose-1,6-diphosphatase and for protein concentration by a modification of the method of Lowry et al. [17]. All values were reported as mean ± S.D.

Rabbits were made diabetic by intravenous injection of alloxan monohydrate (Sigma Chemical Co.). The usual doses were approximately 200 or 300 mg/kg body weight which were administered under ether or pentobarbital anesthesia. Serum was analyzed for glucose by a Beckman Glucose Analyzer on the day of killing which was 3—7 days after administration of alloxan. Diabetes was considered severe when the concentration of glucose exceeded 500 mg%, moderate at 300—499 mg% and mild below 300 mg%.

Rabbits maintained on rabbit chow, were fasted for various periods. The results are presented in Table I. In the livers the concentration of fructose-1,6-diphosphatase increased with fasting for as little as 48 h (differences significant at the 0.95 level as determined by the Student *t*-test). A statistically significant increase in assayable activity, however, was not observed until 96 h of fasting. We chose to express our activity in units/g tissue rather than units/mg protein to allow comparison with data reported by others (i.e. Ref. 3). Since no significant variation in the protein concentration/g tissue was observed (data not shown) in fasting, carbohydrate feeding, protein feeding and diabetes, we felt units/g tissue to be a valid parameter. The specific activity of the fructose-1,6-

TABLE I

VARIATION IN RABBIT FRUCTOSE-1,6-DIPHOSPHATASE CONCENTRATION

At least four animals were used for each category (total = 27). Results are given as mean ± S.D. Fasting followed rabbit chow feeding.

	Rabbit chow	Fast			96 h fast 24 h re-feed	High protein
		48 h	72 h	96 h		
A Liver						
μM	3.4 ± 0.3	4.5 * ± 0.3	4.4 * ± 0.8	4.7 * ± 1.1	4.3 ± 1.0	7.2 * ± 1.4
U/g liver	8.1 ± 1.4	9.3 ± 2.1	8.8 ± 3.3	10.9 * ± 2.2	7.8 ± 1.7	10.2 * ± 1.6
U/nmol enzyme	2.4 ± 0.3	2.1 ± 0.4	2.0 ± 0.7	2.4 ± 0.9	1.8 ± 0.2	1.5 * ± 0.5
B Kidney						
μM	2.6 ± 1.1	2.0 ± 0.6	2.3 ± 1.0	3.2 ± 1.1	3.9 ± 1.4	3.5 ± 1.0
U/g kidney	5.0 ± 0.9	4.9 ± 1.6	4.7 ± 0.5	6.2 ± 1.9	5.8 ± 0.9	4.0 ± 0.5
U/nmol enzyme	2.2 ± 0.8	2.4 ± 0.9	2.4 ± 1.1	2.0 ± 1.0	1.6 ± 0.3	1.2 * ± 0.3

* Significant at the 95% level as determined by Student's *t*-test.

TABLE II

FRUCTOSE-1,6-DISPHOSPHATASE IN ALLOXAN-INDUCED DIABETES

Results are given as mean ± S.D. Four rabbits were killed for each category for a total of 12 rabbits. Mean serum glucose for the moderate group was 373 mg% and for the severe group, 620 mg%.

	Control	Moderate	Severe
Liver			
μM	3.4 ± 0.3	5.0 ± 1.6 *	8.7 ± 1.9 *
U/g	8.1 ± 1.4	10.3 ± 4.2	18.1 ± 4.3 *
U/nmol	2.4 ± 0.3	2.1 ± 0.5	2.1 ± 0.4
Kidney			
μM	2.6 ± 1.1	2.8 ± 0.9	4.7 ± 2.8
U/g	5.0 ± 0.9	5.5 ± 1.1	7.3 ± 2.8
U/nmol	2.2 ± 0.8	2.1 ± 0.6	1.8 ± 0.6

* Significant at the 95% level as determined by Student *t*-test.

diphosphatase as defined as units of activity/nmol of fructose-1,6-diphosphatase did not show any significant changes during fasting. When rabbits that had been fasted for 96 h, were refed chow for 24 h, the molarity, and U/g tissue returned to levels not significantly different from fed animals. Thus the specific activity once again remained the same. When kidney extracts of fasted rabbits were analyzed no statistically significant differences in molarity, U/g tissue or specific activity were observed under any conditions. In the liver the molarity of fructose-1,6-diphosphatase was not significantly altered by either the high-carbohydrate or high-fat when compared to the rabbits fed normal rabbit chow. On the other hand, the concentration of liver fructose-1,6-diphosphatase was more than doubled in rabbits fed a high-protein diet (Table I). The amount of assayable activity was not significantly changed under the high-carbohydrate or high-fat regimens, but increased slightly though significantly under a high-protein diet. The specific activity (U/nmol) of fructose-1,6-diphosphatase, therefore, remained unchanged for high-carbohydrate, and high-fat diets but was significantly decreased under a protein diet. When comparable kidney extracts were analyzed no statistically significant changes in the molarity, assayable activity, or specific activity were observed except under a high-protein diet. Then the assayable activity was slightly decreased and consequently so was the specific activity.

The effect of diabetes upon the concentration of liver enzyme are presented in Table II. The molarity of fructose-1,6-diphosphatase increased with increasing degree of severity of diabetes with a greater than two-fold increase in concentration in the severest cases. Similarly the assayable activity also increased with worsening diabetes. Since both increased in a parallel fashion the specific activity remained the same in all groups. In comparison to the liver, the kidney was relatively less responsive to the diabetic state.

Discussion

Two types of subunit were present in the tetramers of our purified enzyme both before and after labeling with ^{125}I. However, our radioimmunoassay

probably did not distinguish between tetramers containing one form or the other. This is substantiated by the complete inhibition of purified enzyme activity by the antibody (Figs. 2 and 3) which would require recognition of both types of tetramer by the antibody. Since several conditions such as seasonal variation, exposure to cold or fasting [18] increase the proteolytic activity of cytosol fractions, both types of tetramers could be present physiologically in tissues. That the tetrameric form of the enzyme is the species actually being measured is supported by our specific activity figures (Table I). The specific activity of the enzyme in fed rabbit livers is 2.4 units/nmol of enzyme or 16.6 units/mg protein based upon tetrameric molecular weight of 144 000. This figure falls well within range of specific activities reported in the literature (14.6 U/mg [19]; 22.5 U/mg [7]). In fact, the specific activity of the tissue enzyme may represent a minimum since the activity which is measured in tissue extracts is subject to inhibition by other tissue components.

Others have labeled liver fructose-1,6-diphosphatase [20] by the chloramine T method, using only residual enzymic activity as an indication of enzyme integrity. Since assayable activity may be due solely to unlabeled enzyme, we chose chromatographic and electrophoretic methods for detection of enzyme damage. Further, greater sensitivity was obtained by our radioimmunoassay using Bolton-Hunter labeled enzyme (as little as 2 ng was detectable) compared to that of Kolb and Grodsky (80 ng) [20]. Thus our labeled enzyme apparently retained suitable immunologic properties as well.

Effect of Fasting

The molarity of fructose-1,6-diphosphatase in liver increased approximately 1.4 fold after 96 h of fasting (Table I). Similarly, the assayable activity increased a comparable amount under the same fasting conditions as measured under our assay conditions. Pontremoli et al. [3] using a different assay mixture observed a nearly two-fold increase in measurable activity after 96 h fasting. Their normal fed activity was only 4 U/g liver compared to 8.1 U/g by our measurements. To ascertain whether these differences were due to assay conditions, we repeated our assays deleting $(NH_4)_2SO_4$ and the AMP removal system. For normal fed activity, 5.5 U/g (±1.4) was now obtained and after 96 h fasting, 9.3 (±0.95), a 1.6-fold increase. It is obvious that different assay conditions can yield widely varying enzyme activities for the same extracts. The radioimmunoassay for fructose-1,6-diphosphatase is apparently unaffected by the presence of AMP as dialyzed extracts gave the same values as undialyzed extracts. Therefore, in this case, the radioimmunoassay is a more reliable measurement of enzyme concentration.

The potential of the combined use of radioimmunoassays and activity measurements for defining mechanisms of regulation is promising. For example, if both activity and radioimmunoassay measurements increase (or decrease) in parallel, an increase (or decrease) in the quantity of enzyme would be suspected (increased synthesis or degradation). Conversely, if the radioimmunoassay measurement remains constant while activity measurements increase (or decrease) a change in the quality of the enzyme would be indicated (allosteric or covalent modification).

Effect of Diet

We have attempted in our assays for this paper to compensate for two enzyme effectors so that our activity measurements and radioimmunoassay measurements agreed in most cases, the exception being extracts from protein-fed rabbits. Herein, the liver enzyme as measured by radioimmunoassay increased 2.2 fold whereas the assayable activity remained either unchanged, in the absence of an AMP removal system, or increased 1.3 fold, in the presence of an AMP removal system. An unknown inhibitor of activity (i.e. increased nitrogenous waste products) present in these liver extracts can, therefore, be suspected.

Effect of diabetes

The diabetic state produced the most dramatic increases in both concentration and assayable activity of liver fructose-1,6,-diphosphatase (2.5 and 2.2 fold, respectively). The kidney enzyme also showed an increasing trend in concentration and activity in the severest cases but not to a statistically significant degree. In all cases, the increase in enzyme concentration and activity paralleled the increasing severity of the induced diabetes. The high degree of variability, particularly in activity measurements, is related to the variable serum glucose levels obtained. The concentration and activity measurements both varied according to the serum glucose levels.

Since the increases in enzyme concentration and activity paralleled one another, insulin might be envisioned as a repressor of fructose-1,6-diphosphatase synthesis: the more severe the diabetes the less insulin present and the more fructose-1,6-diphosphatase synthesized. The increase in glucoenogenesis, often noted in diabetes, then, could be related to the lack of the repressor, insulin. By extension, one could speculate that during fasting and on the high-protein diet the blood glucose levels and subsequently insulin secretion would drop. With a decrease in repressor substance, more fructose-1,6-diphosphatase would be synthesized. Besides this contribution to increased gluconeogenesis, the role of other factors, such as hyperglucagonemia, must also be evaluated.

The extent to which the cell regulates carbon flux through a regulated enzymic reaction by varying the concentration of that regulatory enzyme can now be assessed. Under multiple conditions tested the rabbit liver cell did vary the concentration of fructose-1,6-diphosphatase. Since the cell did not vary enzyme activity alone, variation in absolute concentration must offer some advantage. Because of intracellular microenvironments and allosteric phenomena, we cannot apply activity assays which unequivocally reflect in vivo enzyme concentration. In this regard the radioimmunoassay can more faithfully provide information about intracellular concentration.

Fasting has been shown to cause a decrease in liver weight of approximately 30% [21,22] whereas the concentration of fructose-1,6-diphosphatase increased by 38%. Presumably, there was only a small absolute increase in total organ enzyme, if any at all, due to fasting. In contrast, liver weight increases or remains unchanged following protein feeding and diabetes [21]. In these conditions the concentration of enzyme increased over 200%. Thus, there were even larger absolute increases in organ enzyme. The ideal experimental situation is to measure the enzyme protein in isolated cells which would permit expression of data on the basis of per number of cells.

Although fructose-1,6-diphosphatase activity followed concentration faithfully under most conditions, in the following paper, we assess another enzyme of carbohydrate metabolism whose assayable activity remained constant while the enzyme concentration varied. Thus, assayable activity measurements cannot in all cases be taken as a reflection of enzyme concentration. The radioimmunoassay for enzymes should come to make a special contribution which cannot be made by assayble activity methods alone.

Acknowledgement

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