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Intracellular Concentration of Hepatic Glycerol-3-phosphate Dehydrogenase in Normal, Diabetic, and Hormonally Manipulated Rabbits (41022)[1]

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Abstract. A radioimmunoassay (RIA) for cytosolic glycerol-3-phosphate dehydrogenase (GPDH) was developed as a means of determining enzyme concentration independent of assayable activity. The GPDH concentration (nmole/g wet wt), activity (units/g wet wt), specific activity (units/nmole GPDH), total liver concentration (nmole/liver/kg body wt), and total liver activity (units/liver/kg body wt) were determined under conditions favorable and unfavorable to gluconeogenesis. Fasted rabbits showed a decrease in total liver nanomoles of GPDH per killigram of body weight. Cortisone, triamcinolone, and thyroxine administration caused increases in total liver units and total liver nanomoles of GPDH per killigram of body weight in the fed animal. The increases or decrease observed correlated directly with changes in liver size. Diabetic, insulin-controlled diabetic, and glucagon-treated fed animals showed neither GPDH activity nor concentration changes. There was no change in the specific activity of GPDH under any conditions. The constant specific activity observed indicates a single catalytic state for the enzyme. Thus, changes in total liver enzyme rather than in specific activity become critical in the regulation of the GPDH reaction.

Several functions have been attributed to cytosolic glycerol-3-phosphate dehydrogenase (EC 1.1.1.8; GPDH). Glycerol-3-phosphate produced by the GPDH-catalyzed reduction of dihydroxyacetone phosphate is used in the biosynthesis of triglycerides and multiple complex lipids. Together with other dehydrogenases, GPDH probably helps maintain a "correct" NAD^+/NADH ratio (1). In many tissues, and perhaps also in liver GPDH contributes to a shuttle process (2–5) whereby cytosolic-derived reducing equivalents in the form of glycerol-3-phosphate, are transported into mitochondria. White and Kaplan (6, 7) have suggested that the reaction may be reversed to supply dihydroxyacetone phosphate for gluconeogenesis. In this paper we describe the development of a specific radioimmunoassay (RIA) for cytosolic GPDH, its successful application to liver, the enzyme activity as a function of concentration, and the activity of the enzyme in the gluconeogenic state. The enzymatic activity (units/g wet wt), the concentration (nmole/g wet wt), the specific activity (units/nmole GPDH), total liver activity (units/liver/kg body wt), and total liver concentration (nmole/liver/kg body wt) were determined for cytosolic GPDH under conditions favorable and unfavorable to gluconeogenesis.

Materials and Methods. Male and female New Zealand rabbits weighing approximately 1200 g were used in this study. Except for the fasted rabbits, all were maintained on a commercial rabbit chow diet until sacrifice. Diabetes was induced by a single intravenous injection of alloxan (100 mg/kg) under anesthesia of ketamine hydrochloride and promazine. Seventy-two hours later animals were sacrificed or insulin therapy was begun. Lente insulin (U-40) and glucagon were obtained from the Eli Lilly Company, cortisone acetate from the Upjohn Company, triamcinolone diacetate from Lederle, and levothyroxine from Flint Laboratories. Animals were weighed, anesthesized in ether, sacrificed by exsanguination, and the livers were removed and weighed. Homogenates (20%, w/v) were prepared in 0.25 *M* sucrose containing 10 m*M* Tris–HCl buffer, pH 7.5, 1 m*M* dithiothreitol, and 0.1 m*M* EDTA using a Polytron apparatus and were centrifuged for 1 hr at 114,000*g*. The supernatant frac-

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tion was assayed for concentration and activity.

Highly purified rabbit muscle GPDH obtained from Boehringer-Mannheim was purified to homogeneity using a Waters Associates Inc. high-pressure liquid chromatograph equipped with an absorbance detector and fitted with two 125-protein analysis columns connected in series. The silica-based 125-protein column has a 7.8 mm i.d., is 30 cm long, and has an exclusion limit of 80,000 daltons. Enzyme samples of approximately 1 mg were dissolved in 0.1–0.2 ml of 50 m*M* sodium phosphate buffer, pH 7.2, containing 0.15 *M* sodium sulfate and chromatographed. Pressures of 400–600 psi were maintained at a flow rate of 1 ml/min. Effluent was monitored spectrophotometrically at 280 nm and by enzyme assay (8). Active fractions were pooled, and the protein content was determined by a fluorescamine procedure (9, 10) and submitted to SDS and anionic polyacrylamide gel electrophoresis. Purified enzyme to be used for ^{125}I labeling was made 1 m*M* in mercaptoethanol and EDTA, divided into aliquots, and stored at −20°. In addition to the mercaptoethanol and EDTA, purified GPDH to be used as standard in competitive binding assay was made 1% in BSA and also stored at −20°.

γ-Globulin against rabbit muscle GPDH was raised in goats as previously described for muscle phosphofructokinase (11). We established that the muscle and liver enzymes are immunologically indistinguishable. Anti GPDH γ-globulin was evaluated against purified enzyme and tissue extracts by Ouchterloney double diffusion. The γ-globulin was also evaluated by tissue enzyme inhibition studies. Cytosolic fractions of liver, muscle, and kidney were incubated with anti-enzyme γ-globulin in 0.4 *M* Tris–HCl buffer, pH 7.6, for 24 hr at 4°, centrifuged at 5000*g*, assayed for enzymatic activity, and compared to control values (nonspecific γ-globulin added). The specific γ-globulin was also tested against the purified enzyme in an analogous manner.

Fifteen micrograms of purified GPDH was labeled with $Na^{125}I$ using the procedure of Bolton and Hunter (12). The reagent, [TAGIT, Calbiochem. Co.] or 3-(4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester (0.8 nmole) was iodinated to a specific activity of approximately 10 mCi/μg. The reaction was conducted at 4° for 15 min. Labeled enzyme and unreacted reagents were separated on a 30 × 1-cm Biogel P-10 column equilibrated with 50 m*M* sodium phosphate, pH 7.5, containing 0.25% (w/v) gelatin. Any fragmented, denatured, or aggregated enzyme was separated from labeled intact dimeric enzyme on a 90 × 1.1-cm Biogel P-150 column equilibrated with 50 m*M* sodium phosphate buffer, pH 7.5, containing 1 m*M* EDTA, 1 m*M* mercaptoethanol, and 0.02% NaN_3. Fractions corresponding to the elution volume of native enzyme were pooled, made 1% in BSA, and tested for antigenicity against the specific γ-globulin. In a volume of 0.5 ml, 25,000–30,000 cpm of labeled enzyme, 0.1 ml buffered 1% ovalbumin, P-150 column elution buffer, and various concentrations of specific γ-globulin were incubated at 4° for 48 hr. A second-stage antibody was used to precipitate the antigen–antibody complex as previously described (11). Reaction mixtures were counted in a Beckman gamma counter, centrifuged at 1520*g* for 10 min, the supernatants were aspirated, the pellets were then counted, and the percentage of original total counts contained in the pellet was calculated. Nonspecific binding was determined as described by omitting the specific γ-globulin.

Competitive binding studies were performed using an amount of specific γ-globulin (0.75–1.50 μg) which precipitated 35–45% of a constant amount of ^{125}I-labeled GPDH (3–5 ng) in the presence of increasing amounts of purified standard enzyme. Nonspecific binding samples (no specific γ-globulin) and control samples (no unlabeled GPDH) were run with each competitive binding study. After 48 hr at 4° second stage was added and incubation continued overnight. Samples were then counted and centrifuged; supernatants were aspirated, and pellets were counted. Bound/free was plotted against nanograms of standard enzyme on log-logit paper (13).

Data processing was as described previously (11, 14, 15).

Rabbit enzymes (100–600 ng) pyruvate kinase, phosphofructokinase, triose phosphate isomerase, aldolase, phosphoglucoisomerase, glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and fructose bisphosphatase were tested in the competitive binding assay for GPDH.

Fifty-microliter aliquots of freshly prepared 114,000*g* supernatant fractions of liver were diluted 10-fold for measurements of enzymatic activity under maximum velocity conditions (8). The same extracts were assayed for enzyme concentration by radioimmunoassay in final dilutions of 1:10,000, 1:20,000, 1:30,000, and 1:40,000 as previously described except that the diluted tissue extracts replaced the standard purified GPDH as enzyme source. Percentage of control values obtained from tissue enzyme was converted to concentrations based upon the standard curve.

Results. The hplc profile for GPDH is shown in Fig. 1A. Enzymatic activity was observed in the major peak absorbing at 280 nm. Single protein stained bands were observed on both nondenaturing anionic polyacrylamide gels (not shown) and SDS polyacrylamide gels (Fig. 2). The stained band of enzyme protein coincided exactly with label in the gel. The specific activity of purified enzyme was 130 units/mg.

One milligram of specific γ-globulin inhibited all of the enzyme activity in 50-μl aliquots of 20% homogenates of kidney (1.8 μg enzyme), liver (3.9 μg enzyme), and 93% of the activity in muscle (3.5 μg enzyme). Double-diffusion studies of cytoplasmic extracts of the three tissues against anti-enzyme γ-globulin each showed a single precipitin line identical to the line observed with purified GPDH (Fig. 3) Double-diffusion studies of anti-enzyme γ-globulin against the combined extracts from the three tissues and the purified enzyme also showed only one continuous precipitin line (not shown).

A Biogel P-150 chromatographic profile of iodinated GPDH is presented in Fig. 1B. Fractions 24–30 were pooled for use in the RIA. Immunological affinity of the specific γ-globulin for ^{125}I-GPDH is demonstrated in Fig. 1C. Greater than 90% of the radiolabeled enzyme was bound by the specific γ-globulin. Competition for antibody between a constant amount of ^{125}I-GPDH (3–5 ng) and increasing amounts of standard enzyme for a fixed amount of specific γ-globulin (0.75 μg) are demonstrated in Figure 1D.

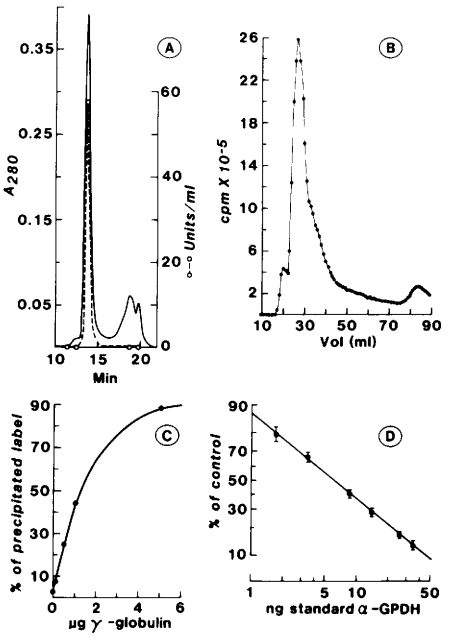

FIG. 1. (A) hplc profile of GPDH. Aliquots of GPDH (0.5–1.0 mg) were chromatographed at a rate of 1 ml/min on a Waters hplc fitted with two 125-protein columns. (B) Biogel P-150 profile of ^{125}I-GPDH. Fractions 24–30 which corresponded precisely with the chromatographic behavior of native GPDH, were pooled and used as the ^{125}I-GPDH source for all subsequent studies. (C) Titration of ^{125}I-GPDH with specific GPDH γ-globulin. To a fixed amount of ^{125}I-GPDH (3–5 ng containing 25,000–30,000 cpm) was added increasing amounts of specific γ-globulin. (D) Competitive binding plot for GPDH. The extent of competition between iodinated and standard GPDH for specific GPDH γ-globulin was demonstrated with each analysis of tissue extract. Each point represents the mean of 10 individual values and the error bars denote the standard deviation of the means. Standard curves were plotted from the results of linear regression analysis using a computer program.

The inclusion of various other rabbit en-

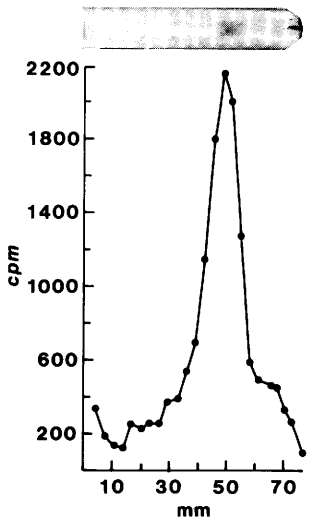

FIG. 2. SDS polyacrylamide gel profile of labeled and unlabeled GPDH. Eight-microgram of purified GPDH were mixed with an equal volume of 2% SDS and 10 *M* urea. The samples were boiled for 5 min, cooled, and electrophoresed at 2.5 mA in 5.6% polyacrylamide gels (4 × 75 mm) at pH 8.3. Gels were stained for protein using 0.025% Coomassie R-250 and destained in 7% acetic acid. ^{125}I-GPDH samples after P-10 chromatography were subjected to the same procedure. Following electrophoresis the gels were sliced into 2.5-nm sections and counted for radioactivity. After correction for background 93% of radioactivity was found in fractions 10–20.

zymes in competitive binding studies had no effect on the percentage of radioactivity precipitated by constant amounts of specific GPDH γ-globulin and ^{125}I-GPDH either in the presence or absence of unlabeled GPDH.

Enzyme activity data from kinetic measurements and concentration data from radioimmunoassays are listed in Table I. Total liver activity (units/liver/kg body wt) and total liver concentration (nmole/liver/kg body wt) were statistically higher for triamcinolone-, cortisone-, and thyroxine-treated animals. The opposite response in concentration (nmole/liver/kg body wt) was elicited for 4-day fasted rabbits. Even though differences occurred in liver activity and concentration no changes in specific activity (units/nmole GPDH) were observed in the study.

Discussion. Through the combined use of RIA and enzymatic assay we have demonstrated, for the first time, direct correlation between concentration and activity for rabbit liver GPDH. This is not the case for some other enzymes of carbohydrate metabolism (14–16). The results of this work were totally dependent upon the successful development and application of the radioimmunoassay to liver cytosol. The specific activity of purified enzyme was 10.1 units/nmole (130 units/mg) and this compares favorably with our experimental values, e.g., 9.2 for control livers. The introduction of radiolabel into an enzyme as antigen and its use in a competitive binding assay affords distinct advantages over immunotitration (17) and radial immunodiffusion techniques (18). The mass of enzyme can be measured independently of activity. Thus, the mass of immunologically active but enzymatically inactive enzyme is detectable. In addition, competitive binding assays are sufficiently sensitive to measure nanogram quantities of enzyme.

Although rabbit liver and muscle GPDH exhibit differences in amino acid composition (4), antibody to either enzyme cross-reacts (19) due to similarities (in primary structure) between isozymes. In the rabbit two immunologically distinguishable forms have been detected; one form predominates in liver, kidney, and muscle while the second is found to predominate in heart (19). The anti-muscle GPDH antibody developed in our laboratory also exhibits cross-reactivity toward kidney and liver GPDH. Because there is only one predominating isozyme in rabbit liver the development and application of RIA for GPDH was simplified. In contrast multiple molecular forms of GPDH are observed in young rats (20) which would result in considerable complexity if a single antibody had been developed from a heterogeneous population of GPDH isozymes. For those cases, the isozymes would first have to be separately purified, individual antibodies developed, and an absence of cross-reactivity demonstrated before any data regarding concentration, enzyme activity, and specific activity changes could be presented. Similarly, multi-subunit enzymes under allosteric control, such as phosphofructokinase (11),

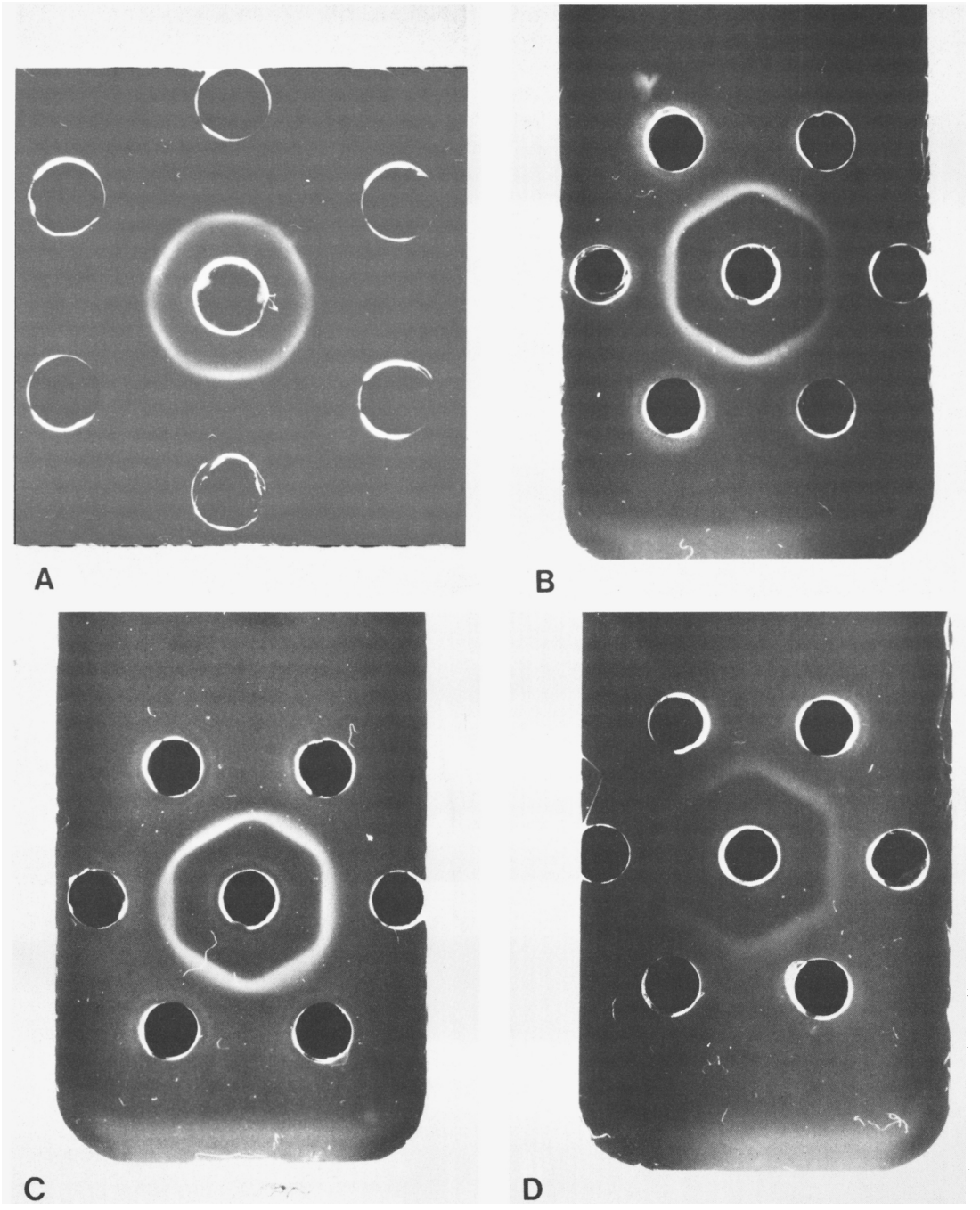

FIG. 3. Double-diffusion patterns of purified and tissue GPDH against specific GPDH γ-globulin. All center wells contained 625 μg of specific GPDH γ-globulin while the surrounding wells contained (A) 2 μg of purified GPDH or 5-μl cytoplasmic extracts of 20% tissue homogenate; (B) liver; (C) muscle; (D) kidney.

TABLE I. LIVER CYTOSOLIC GLYCEROL-3-PHOSPHATE DEHYDROGENASE

Type	*N*	Animal wt (g)	Liver wt (g)	Units/liver/kg body wt	μM (μmole / kg wet weight)	nmole/liver/kg body wt	Units/nmole GPDH (S.A.)
Control	(12)	1296	43	1727	5.1	192	9.2
		±	±	±	±	±	±
		203	18	437	0.8	50	1.8
Triamcinolone	(11)	1298	79*	2571*	4.5	258*	10.4
		±	±	±	±	±	±
		133	27	409	1.3	64	1.7
Cortisone	(6)	1280	71*	2585*	5.6	310*	8.3
		±	±	±	±	±	±
		171	19	770	0.6	74	1.1
4-day Fasted	(6)	1325	36	1395	4.9	130*	11.3
		±	±	±	±	±	±
		140	6	232	0.4	17	2.0
Thyroxine	(6)	1456	74*	2494*	5.3	273*	9.4
		±	±	±	±	±	±
		90	9	267	0.6	46	1.7
Glucagon	(14)	1282	61	2042	5.3	240	8.7
		±	±	±	±	±	±
		212	23	496	0.9	69	1.6
Diabetes	(18)	1119*	46	2032	5.3	214	10.0
		±	±	±	±	±	±
		159	9	419	1.3	61	2.6
Insulin-Treated Diabetes	(17)	1528*	73*	2170	4.9	238	9.1
		±	±	±	±	±	±
		200	13	382	0.9	57	1.9

* Significant at 95% confidence level with control as reference; Student's *t* test was used for statistical analysis.

Note. Numbers in parentheses signify animals used. Data are mean ± standard deviation. Insulin (2 units/day) was administered subcutaneously for 4, 8, 10, or 15 days before sacrifice. There were at least four animals in each treatment group. Glucagon (1 mg) was injected subcutaneously every 6 hr plus 1 hr prior to sacrifice. Thyroxine (20 μg) and triamcinolone (4 mg) were injected intramuscularly each day for 3 days.

pyruvate kinase (14), and fructose bisphosphatase (15) often require special conditions for the successful application of a specific RIA.

Hepatic cytosolic GPDH is not known to be under allosteric control. Control of the reaction *in vivo* would have to be vested in the catalytic state of the enzyme as perhaps influenced by covalent modification, its concentration in liver and the availability of substrate. How is control exerted? We have shown that specific activity remains constant (units/nmole GPDH). On the other hand enzyme mass was variable. Although nanomoles of GPDH per gram of wet weight liver did not change, nanomoles of GPDH per liver per killigram of body weight changed greatly secondary to changes in liver mass (Table I).

An increase in liver mass could be due mainly to hyperplasia. This would then result in the *de novo* synthesis of enzyme obligated to the growth and development of new liver cells. A less plausible hypothesis is that induction of enzyme occurred in preexisting cells. The application of our radioimmunoassay to a constant number of isolated hepatocytes in culture might help make the distinction.

Does GPDH play a role in gluconeogenesis? Although the enzyme is gluconeogenic for animals maintained on a glycerol diet (21–23) we found that fasting, diabetes and glucagon administration—all known to favor gluconeogenesis—did not increase units GPDH per liver per killigram of body weight, nanomoles of GPDH per liver per killigram of body weight, or units per nanomole of GPDH.

This work establishes that the liver maintains GPDH in a single catalytic state, that unlike other enzymes of carbohydrate metabolism (14–16) the activity directly follows the concentration, that total liver GPDH activity is determined by liver size and not by a change in specific activity, and that no evidence was found for increased GPDH activity under gluconeogenic conditions.

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