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Quantification of liver and kidney phosphofructokinase by radioimmunoassay in fed, starved and alloxan-diabetic rats

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A newly developed specific radioimmunoassay was used to quantify phosphofructokinase protein directly and independently of assayable activity in liver and kidney cytosol of normal fed, starved and alloxan-diabetic rats. In the fed state, liver phosphofructokinase concentration was 0.096 μM and the kidney enzyme was 0.086 μM (μmol/kg of tissue). In the starved state (24h), liver and kidney phosphofructokinase concentrations decreased by 30%. Prolonged starvation up to 72h did not further decrease enzyme concentration. In liver, total enzyme content during starvation declined by more than 50%, secondary also to a decrease in liver weight. In the alloxan-diabetic rats, there was a 22% decrease in enzyme protein concentration in liver and kidney. Total enzyme content per liver actually decreased much more (46%), because diabetes also resulted in a decrease in liver size. In conjunction with assayable activity measurements, the results of the radioimmunoassay allowed us to calculate the apparent specific activity of the enzyme. The specific activity of the kidney enzyme was 2–3 times that of the liver. Little or no change in specific activity of the liver or kidney enzyme occurred as a result of starvation or chemically induced diabetes. Tissue enzyme concentrations of phosphofructokinase unequivocally reconcile the ultimate results of changing rates of synthesis and degradation and are useful data in the design of spectrophotometric, kinetic, aggregation–disaggregation and other studies.

Liver and kidney contain isoenzymes of phosphofructokinase (EC 2.7.1.11) that catalyse the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate, the rate-limiting reaction of glycolysis (Randle *et al.*, 1966; Dunaway & Weber, 1974*a*; Uyeda, 1979). Liver tissue contains the L form of the enzyme, whereas kidney contains primarily L form plus some isoenzymes not present in liver.

Regulation of the enzyme is known to be complex and involves allosteric phenomena, possibly in combination with aggregation–disaggregation phenomena (Reinhart & Lardy, 1980*c*). Liver and kidney phosphofructokinase assayable activities have been shown to vary in response to starvation and diabetes mellitus (Dunaway *et al.*, 1978; Dunaway & Weber, 1974*b*). Assayable activity is associated with the tetrameric enzyme and aggregates thereof, but not with monomer or dimers of the enzyme (Paetkau & Lardy, 1967). Although there have been numerous kinetic studies, no study up to the present one has investigated tissue concentration values by RIA in order to determine to what extent adaptive phenomena might also operate to control the flux of carbon in the phosphofructokinase reaction.

Immunoprecipitation techniques have been employed to measure the amount of enzyme present in the liver (Dunaway & Weber, 1974*b*) and to study the effect of phosphorylation and dephosphorylation on enzyme activity (Brand & Söling, 1975). However, one of the major problems associated with these techniques has been a lack of sensitivity; only microgram quantities of enzyme protein could be measured. The use of immunotitration techniques to quantify an enzyme, whereby a measured immune serum is used to eliminate the activity per unit weight of tissue, suffers the disadvantage of being dependent on an activity

Abbreviation used: RIA, radioimmunoassay.

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assay. In addition to greater sensitivity, RIAs measure the enzyme protein directly, independent of activity.

In the present paper we report the development of an RIA for rat liver phosphofructokinase and its application as a direct and independent measurement of the intracellular concentration of phosphofructokinase in fed control, starved and alloxan-diabetic animals. We have demonstrated by direct measurement of the enzyme protein that the enzyme is subject to adaptive changes even though it is also among those most sensitive to allosteric modifiers.

Materials and methods

Animals

Male Sprague–Dawley rats (approx. 220g body wt.) were maintained on a standard Purina diet with free access to water at all times. Lighting was controlled so as not to interfere with the nocturnal habits of the animals. Rats were subjected to various dietary and hormonal states before analysis. For the starved animals, food was removed at 4, 8, 12, 24, 48 or 72h before they were killed. Diabetes was induced by a single intravenous injection of alloxan monohydrate (60mg/kg body wt.; 20mg/ml in 0.9% NaCl; Vega Biochemicals, Tucson, AZ, U.S.A.). Then 72h later animals were stunned and then killed by decapitation. Diabetes was confirmed by a blood glucose value of greater than 300mg/100ml as determined by Chem Strips (Boehringer Mannheim, Indianapolis, IN, U.S.A.).

Preparation of cytosol

Livers and kidneys were excised, washed in cold 0.9% NaCl, weighed and homogenized in 4vol. of buffer [0.25M-sucrose/10mM-Tris/HCl (pH8.0)/30mM-KF/1mM-2-mercaptoethanol/0.1mM-EDTA]. The cytosolic fraction was obtained by centrifugation at 110000*g* for 1h at 4°C and was used for both activity assays and RIAs.

Enzyme purification and assay of rat liver phosphofructokinase

Rat liver phosphofructokinase was purified as described by Reinhart & Lardy (1980*a*) and assayed with a glycerol-3-phosphate dehydrogenase-coupled reaction system that followed the decrease in NADH absorbance at 340nm (Hansen & Veneziale, 1980). The final concentrations of fructose 6-phosphate and KCl were changed to 4mM and 100mM respectively, and the pH of the buffer was increased to 8.0. One unit of activity represents 1μmol of fructose 1,6-bisphosphate formed/min at 25°C.

Antibody preparation and characterization

The antiserum was shown to be specific for phosphofructokinase by Ouchterlony double-diffusion studies (Hansen & Veneziale, 1981; Ouchterlony & Nilsson, 1978). Methods for preparation and evaluation of antibody specificity and tissue inhibition studies have been described previously (Veneziale *et al.*, 1981*b*,*c*).

Radioiodination of phosphofructokinase

Purified enzyme was labelled with ^{125}I by the lactoperoxidase procedure (McLaughlin & Pitot, 1976; Palacios *et al.*, 1972). Free iodine was removed by $(NH_4)_2SO_4$ fractionation. Approximately one ^{125}I atom was incorporated for every 8–12 tetrameric enzyme molecules. Iodination of phosphofructokinase with Bolton–Hunter reagent (Bolton & Hunter, 1973) or direct iodination by the chloramine-T procedure always yielded multiple enzyme fragments of phosphofructokinase. Labelling of phosphofructokinase with ^{125}I to a higher specific radioactivity with lactoperoxidase decreased the maximal amount of antigen precipitated by antibody. The labelled enzyme was purified from enzyme fragments by Bio-Gel A column chromatography (Hansen & Veneziale, 1980). Radiolabelled enzyme stored at 4°C could be utilized in the RIA for a period of 4–7 weeks.

RIAs: standard competitive binding curves

Assays were conducted in a final volume of 1ml. Standard competitive binding curves were obtained for the RIA by incubating a constant amount of ^{125}I-labelled enzyme (20000–25000 c.p.m.; approx. 12ng of phosphofructokinase) with increasing amounts of unlabelled enzyme (0–320ng) and a constant amount of antiserum sufficient to precipitate 40–50% of the labelled antigen. The final volume was made to 1.0ml with egg albumin buffer [0.1M-sodium phosphate buffer (pH7.5)/1% ovalbumin/0.02% NaN_3] (Mazzotta & Veneziale, 1980). All reaction mixtures were produced in duplicate and maintained at 4°C for 48h. The second-stage incubation mixture consisted of 50μl of a diluted (1:50) goat normal serum and 50μl of rabbit anti-goat serum in each reaction mixture. Total radioactivity and radioactivity remaining in the pellet after centrifugation and aspiration were counted. Data were plotted and analysed as described previously (Veneziale *et al.*, 1981*b*,*c*).

Cytosolic samples were assayed in the same way except that various volumes of diluted cytosol (1:10, 1:100 and 1:1000) replaced standard enzyme.

Results

Antibody evaluation

Antibody to phosphofructokinase showed a single precipitin line of complete identity with purified enzyme, and extracts of liver and kidney, confirming that antibodies were raised only to the enzyme protein of interest (Ouchterlony & Nilsson, 1978).

Inhibition of enzyme activity by antiserum

When purified phosphofructokinase (0.6 unit) was incubated at 24h at 4°C in a final volume of 1 ml with 0, 5, 10, 15, 20, 25 and 50 μl of antiserum, only 10 μl of antiserum was sufficient to remove all of the enzyme activity after centrifugation. The mere union of antibody to enzyme resulted in a decrease in enzyme activity. The addition of 20 μl of antiserum without centrifugation resulted in a greater than 50% decrease of enzyme activity relative to non-immune controls. When non-immune serum up to 25 μl was added, there was no loss of enzyme activity.

Behaviour of ^{125}I-labelled phosphofructokinase

The purification of labelled enzyme on Bio-Gel A (Bio-Rad Laboratories, Richmond, CA, U.S.A.) resulted in a single major peak of radioactivity that was eluted at the volume corresponding to that of unlabelled phosphofructokinase. Enzyme fractions were pooled and diluted in egg albumin buffer for use in the RIA. The labelled phosphofructokinase was further evaluated by testing the ability of specific antisera to precipitate the labelled enzyme. Antibody saturation studies showed that a maximum of 95% of the labelled phosphofructokinase was precipitated by 2.5 μl of a 1:10 dilution of antisera, and 40–50 μl of a 1:5000 dilution of antiserum was necessary to precipitate approx. 50% of the labelled antigen.

When the ^{125}I-labelled enzyme was submitted to sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis (Weber & Osborn, 1969), the major band of radioactivity corresponded to the elution position of the unlabelled monomeric subunit of purified standard phosphofructokinase. Immediately after purification of native phosphofructokinase, electrophoresis in the presence of sodium dodecyl sulphate showed a single band of protein. However, after 3 or more weeks of storage at 4°C, electrophoresis of phosphofructokinase disclosed new bands at or near the top of the gel, representing aggregated subunits.

Fig. 1 shows a composite standard curve generated by combining individual standard data from four separate experiments. The effective range of these assays was 5–500ng of phosphofructokinase. Liver cytosol displaced the standard

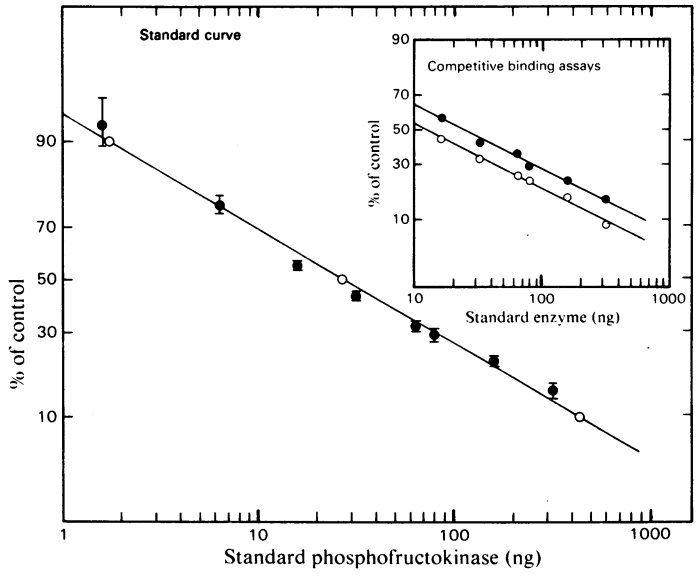

Fig. 1. *RIA standard curve for the analysis of tissue enzyme concentration*

The mean (●) and standard deviation (error bars) of eight values for each amount of purified enzyme are shown. Linear-regression analysis provided the line of best fit (○). Inset: the effect of liver extract on a standard competitive binding curve. No tissue extract was present in control tubes (●). To assess the effect of added liver extract on a standard competitive binding curve, 200 μl of a 1:100 dilution of liver cytosol was added to each reaction mixture containing the indicated amount of standard enzyme (○).

curve to the left in a parallel manner consistent with the presence of endogenous phosphofructokinase (see inset). The same result occurred when kidney cytosol was used. Standard curves remained unaltered when cytosol lacking liver phosphofructokinase was added to the incubation mixtures. Therefore cytosolic substances do not appear to interfere with the RIA. To provide additional evidence for the specificity of the antibody preparation, pyruvate kinase, fructose-1,6-bisphosphatase, triosephosphate isomerase, malate dehydrogenase and glycerol-3-phosphate dehydrogenase were substituted for unlabelled phosphofructokinase; no competition was observed with the labelled phosphofructokinase.

Application of the RIA

In the liver of fed control rats, phosphofructokinase concentration was 0.096 μM, which decreased by 25–30% after starvation for 24–72h (Table 1). After starvation for 48h, the enzyme concentration had declined to 0.072 μM. Total liver enzyme concentration was also shown to decrease significantly ($P \leqslant 0.001$) from 1.09nmol of enzyme/liver in fed rats to 0.51nmol/liver after starvation for 24h. By 48 or 72h of starvation, no further decline of total liver enzyme content was noted. A 32–40% decrease in liver weight, together with the decrease in enzyme concentration, caused a greater than 50% decrease in total enzyme mass. Enzyme activity in units/g did not significantly change as a result of starvation; however, there was a decline in total liver enzyme activity. We cannot be certain that the specific activity of the liver enzyme did change significantly because of starvation (see Table 1), especially since there was no change in diabetes that mimics the starved state (Table 3).

In kidney tissue, phosphofructokinase concentration decreased by approx. 35% after 24h of starvation (Table 2). Kidney enzyme concentration was slightly lower (0.086 μM) than that in liver. Since there was little change in kidney weight with starvation, total kidney enzyme content decreased to about the same extent as enzyme concentration.

Table 1. *Rat liver phosphofructokinase*

Sprague–Dawley rats were subjected to the starvation conditions indicated. Enzyme protein concentration (μmol/kg wet wt. of liver) was determined by RIA. Activity in units/nmol of phosphofructokinase represents the enzyme specific activity based on M_r 328000 for the tetrameric form of the enzyme. Total liver enzyme (nmol/liver) and total liver activity (units/liver) were obtained by multiplying liver weight by the concentration and units per g of liver respectively. Fed animals and their livers weighed 272±16.2g (7) and 11.46±1.10g (7) respectively; 24h-starved animals and their livers weighed 253±5.35g (4) and 7.83±0.47g (4) respectively; 48h-starved animals and their livers weighed 229±3.11 (5) and 6.82±0.16g (5) respectively; 72h-starved animals and their livers weighed 228±10.28g (4) and 6.70±0.66g (4) respectively. Data are presented as means±S.D. (*n*). Under 'Condition' the numbers of rats used are given in parentheses. *, ** and *** stand for $P \leqslant 0.05$, $P \leqslant 0.005$ and $P \leqslant 0.001$ respectively, as calculated by Student's *t* test.

			Activity		
Condition	Concn. (μM)	Liver content (nmol/liver)	(units/g of liver)	(units/liver)	(units/nmol of enzyme)
Fed control (4–7)	0.096±0.004	1.09±0.08	2.48±0.44	28.7±7.1	26.1±5.5
24h-starved (4)	0.066±0.004***	0.51±0.03***	2.52±0.19	19.8±1.9*	38.5±4.0*
48h-starved (5–9)	0.072±0.004***	0.49±0.03***	2.44±0.26	13.5±0.6***	34.0±3.4
72h-starved (4)	0.075±0.003***	0.50±0.04***	2.32±0.23	15.5±0.9**	30.8±2.8

Table 2. *Rat kidney phosphofructokinase*

Animal weights are given in Table 1. Kidneys of fed control rats weighed 2.22±0.24g (12), while 24h-, 48h- and 72h-fasted animals had kidney weights of 2.29±0.08g (4) 1.77±0.10g (5) and 1.93±0.05g (4), respectively. Results are given as means±S.D. *, ** and *** stand for $P \leqslant 0.05$, $P \leqslant 0.005$ and $P \leqslant 0.001$ respectively, as calculated by Student's *t* test.

			Activity		
Condition	Concn. (μM)	Kidney content (nmol/kidney pair)	(units/g of kidney)	(units/kidney pair)	(units/nmol of enzyme)
Fed control (8–12)	0.086±0.018	0.188±0.026	5.51±1.11	10.9±3.7	73.9±9.9
24h-starved (4)	0.056±0.007**	0.129±0.014***	4.95±0.25	11.4±0.8	89.4±17.0
48h-starved (5–10)	0.057±0.008***	0.109±0.009***	5.39±0.50	9.2±1.1	84.7±10.9
72h-starved (4)	0.068±0.004*	0.130±0.010***	5.69±0.26	11.0±0.3	84.4±7.2

Table 3. *Rat phosphofructokinase in alloxan-induced diabetes*

See the legend to Table 1. Animal and liver weights for control rats are reported in the legend to Table 1. Animal and liver weights for the alloxan-diabetic rats were 199.4±6.63g (7) and 7.96±0.85g (7), and kidney weights (per pair) were 2.10±0.08g (7). Chemical induction of diabetes was achieved by a single injection of alloxan monohydrate (60mg/kg body wt.). Diabetics were killed after 72h. Blood glucose values were all above 300mg/100ml for alloxan-diabetic animals. Statistical analysis was based on comparison with fed rats. *, ** and *** represent $P \leqslant 0.05$, $P \leqslant 0.005$ and $P \leqslant 0.001$ respectively.

				Activity		
	Condition	Concn. (μM)	Organ content (nmol/organ)	(units/g of organ)	(units/organ)	(units/nmol of enzyme)
Liver	Control (4–7)	0.096±0.004	1.09±0.08	2.48±0.44	28.7±7.9	26.1±5.5
	Diabetic (4)	0.073±0.012*	0.59±0.10***	1.97±0.12*	15.9±2.2**	27.3±3.8
			(nmol/kidney pair)			
Kidney	Control (8–12)	0.086±0.018	0.188±0.026	5.51±1.11	10.9±3.7	73.9±9.8
	Diabetic (6–7)	0.069±0.015*	0.130±0.032**	5.41±0.65	11.4±0.9	87.5±12.3*

In kidney tissue, neither assayable activity nor specific activity showed any statistically significant difference. The specific activity of the enzyme in kidney tissue was 2–3-fold greater than that of the liver enzyme.

In the alloxan-diabetic animal the enzyme protein concentration decreased in both liver and kidney (Table 3). However, owing to the large decrease in liver weight, the total enzyme content per liver showed a greater decrease. We do not believe that the specific activity of the enzyme in the diabetic differed significantly from that of the normal animal. A slight decrease in assayable activity was noted for liver phosphofructokinase as measured in units/g; the decrease per liver appeared relatively greater because of the concurrent fall in liver weight. Diabetes did not alter the specific activity of the liver enzyme.

Half-life of phosphofructokinase in response to starvation

Animals were starved and killed at 4h intervals. Enzyme mass was assumed to decay exponentially. A plot of the data on semi-log paper allowed calculation of the biological half-life for kidney phosphofructokinase, which was 5.6h. A similar analysis for the liver enzyme gave a half-life of 2h.

Discussion

We have developed and applied RIAs for multiple enzymes of carbohydrate formation and utilization in mammalian tissues in order to measure enzyme protein concentration independently of conventional assayable activity measurements (Donofrio *et al.*, 1981; Veneziale *et al.*, 1981*a*; Moore *et al.*, 1982; Hansen *et al.*, 1983). In the present paper, we report on the quantification of rat liver and kidney phosphofructokinase, the eighth enzyme in the series, also by RIA. We minimized, if not prevented, aggregation by conducting the RIA under dilute conditions and by including 30mM-KF in the homogenization buffer (Ling *et al.*, 1966). KF was included also to inhibit the activity of phosphofructokinase phosphatase, which might have altered the quaternary structure of the native enzyme (Söling *et al.*, 1977). The conditions chosen throughout favoured the maintenance of the enzyme in tetrameric form (Paetkau & Lardy, 1967; Reinhart & Lardy, 1980*a*).

The validity of the RIA was confirmed in several ways. We investigated, but could not establish, whether tissue components interfered with the assay. Documentation further rested on agreement between the specific activity of the enzyme in liver extracts and that of the enzyme purified to homogeneity. The specific activity of the liver enzyme has been reported to be 84–95 units/mg (Dunaway & Weber, 1974*a*; Brand & Söling, 1974; Uyeda, 1979; Reinhart & Lardy, 1980*a*). We found values of 26.1–38.5 units/nmol (Table 1), which correspond to 80–118 units/mg of enzyme.

One may speculate that the presence of mixed kidney L/M phosphofructokinase enzyme molecules could have resulted in lower concentration values if the hybrid tetramers competed less efficiently with the labelled homotetrameric L type in the RIA. This would have contributed to the greater specific activity noted for the kidney enzyme. However, even if 35–50% of the kidney phosphofructokinase were not competing at all in the RIA, the specific activity would still have been 43–57 in the diabetic rat instead of 87 (see Table 3). The immunological studies by Dunaway (1983) support the view that phosphofructokinase isoenzymes exist in part as hybrids in tissues that contain both the L and the M subunits. Taketa (1973) reported the existence of three kidney

isoenzymes: the L type, the M type and a third type that had chromatographic and electrophoretic properties intermediate between those of the major isoenzymes found in liver and muscle. Nevertheless, the presence of hybrid phosphofructokinase isoenzymes in rat kidney tissue remains somewhat controversial. In human kidney the enzyme exists exclusively as the homotetrameric liver form (Etiemble *et al.*, 1979). Chromatography of a mixture of rat liver and muscle extracts did not result in the spontaneous formation of intermediate peaks, which would have indicated L/M hybrids (Tanaka *et al.*, 1971). Our polyclonal antibodies were raised to the L type and gave no reaction at all with muscle tissue extracts. The antibodies reacted with phosphofructokinase from liver and kidney tissues to give a single continuous precipitin line, making no evident distinction between the homogeneous L type of liver and kidney and the putative L/M hybrids of kidney. Furthermore, immunotitration of phosphofructokinase assayable activity in kidney tissue extracts resulted in the ready removal of 94% of the enzyme activity. Thus the hybrid phosphofructokinase molecules present in the kidney, if indeed they exist in significant quantity, were precipitated with similar efficiency as the homotetrameric liver enzyme was precipitated. These findings argue that the RIA detects L-containing hybrids as well as the homotetrameric L form. The higher specific activity of the kidney enzyme(s), except for that which can be attributable to isoenzymes containing the M subunit, cannot otherwise be explained. Kasten *et al.* (1983) reported a specific activity for the purified rat homotetrameric M form of 265 units/mg as compared to our apparent values for the kidney of 189–225 units/mg. A relatively high specific activity for purified kidney phosphofructokinase has also been reported by others (Massey & Deal, 1973).

Dunaway & Weber (1974*b*) reported, on the basis of total liver activity, that 'the major liver phosphofructokinase isozyme' decreased by 60–70% in 6-day-starved rats and in diabetic rats. Neither activity data per g of liver nor total liver weights under the different experimental conditions were provided. Thus a direct comparison with our own activity data is not possible. From pulse-labelling and immunoprecipitation data, Dunaway & Weber (1974*b*) concluded that degradation of phosphofructokinase was accelerated during starvation and that enzyme synthesis was enhanced and degradation decreased during refeeding. Such findings indeed indicated that phosphofructokinase is an adaptive enzyme. However, until the present study the enzyme protein concentration has never been measured directly in either liver or kidney. Only by measuring the coded gene product directly can the final effects of changing rates of synthesis and degradation be reconciled.

The concentrations that we found would allow for phosphofructokinase to exist in an aggregated state intracellularly (Reinhart & Lardy, 1980*b*). We found no support for regulation of kinetic behaviour by stable covalent modification, because we could detect little or no change in specific activity. Although the mechanism(s) controlling the regulation of this enzyme during starvation and/or diabetes are complex, that the enzyme has been shown to be adaptive to these conditions by change in concentration does provide a new level of understanding. Change in total liver enzyme mass was large, owing to a concurrent decrease in enzyme concentration and liver weight, and is important in determining total organ capacity to carry out glycolysis or gluconeogenesis.

In the transition from the fed to the starved state, dramatic reciprocal changes in total liver pyruvate kinase and phosphoenolpyruvate carboxykinase have been shown to take place (Johnson & Veneziale, 1980; Moore *et al.*, 1982). Total liver phosphoenolpyruvate carboxykinase increased 2-fold while at the same time liver pyruvate kinase mass decreased by half. Whatever other regulation this potential futile cycle undergoes, for example by glucagon, the adaptive changes are of considerable magnitude in favour of gluconeogenesis. For the potential cycle involving phosphofructokinase/fructose bisphosphatase, the data differed. Like pyruvate kinase, the total phosphofructokinase mass did decrease (Table 1). Unlike phosphoenolpyruvate carboxykinase, the concentration of fructose bisphosphatase remained unchanged in rat and mouse liver (Zalitis & Pitot, 1979; Hansen *et al.*, 1983); in fact, total liver fructose bisphosphatase mass actually decreased by 25% (Hansen *et al.*, 1983). Therefore, members of this potential futile cycle do not undergo reciprocal adaptive changes. With respect to adaptive changes, control seems to be vested only in phosphofructokinase.

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