Hormonal Regulation of Liver Pyruvate Kinase Concentration and Activity†

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ABSTRACT: The hormonal control of rabbit liver (L type) pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) concentration and activity was investigated. A specific radioimmunoassay was developed to measure the amount (nanomoles) of enzyme independent of the enzyme's activity. From the results the specific activity (units per nanomole) was calculated. Livers were weighed so that total nanomoles of enzyme and units of activity could be evaluated. Liver maintained the enzyme concentration (nanomoles per gram of liver) within narrow limits. The concentration decreased slightly after fasting and the administration of glucagon or triamcinolone. The total organ amount (nanomoles) of enzyme changed but mainly because of changes in liver weight. Liver pyruvate kinase from rabbits fed a control diet which was 50–60% carbohydrate had a specific activity of 12.7 units/nmol of enzyme. Starvation and glucagon each lowered the specific activity to 10.2 units/nmol ($p < 0.005$). Alloxan diabetes also resulted in a decrease which could be reversed by insulin therapy. Triamcinolone decreased the enzyme specific activity to 7.4 units/nmol. Thyroxine caused the enzyme to have a slightly higher value of 14.0 units/nmol. These changes in specific activity resulted mainly from altered enzyme activity (units per gram of liver). Total organ activity was reduced in fasting and after triamicinolone administration. Primarily this was due to a decrease in liver weight and enzyme activity (units per gram of liver) in fasting and to a decrease in enzyme activity after triamcinolone administration. In both states a decrease in enzyme concentration also contributed. We conclude that regulation of liver pyruvate kinase is mainly through regulation of the specific activity, i.e., the catalytic state, of the enzyme. However, in the assessment of total hepatic glycolysis and gluconeogenesis, changes in total organ enzyme activity based on changes in organ weight must also be considered.

Liver contains two main isozymes (M type and L type) of pyruvate kinase (EC 2.7.1.40) (Tanaka et al., 1967; Osterman & Fritz, 1973). The L type has been extensively studied in rat, and its activity is known to be regulated by diet (Krebs & Eggleston, 1965) and hormones (Weber et al., 1965; Feliú et al., 1976; Veneziale et al., 1976). Since the work of Ljungström et al. (1974), a phosphorylation–dephosphorylation reaction, particularly in response to glucagon (Riou et al., 1976), has been the widely accepted mechanism controlling the enzyme activity. Recently, it has been shown (Hall et al., 1979) that L-type pyruvate kinase can undergo a proteolytic cleavage which produces a less active enzyme molecule. They speculate that the cleavage is a secondary response to phosphorylation and irreversibly inactivates the enzyme.

Some of the studies involving the phosphorylation of L-type pyruvate kinase have in part relied upon immunoprecipitation techniques (Riou et al., 1978; Ishibashi & Cottam, 1978) which were adapted to measure the amount of enzyme present in the liver. This permitted calculation of specific activity (units per enzyme mass) (Kohl & Cottam, 1976; Hopkirk & Bloxham, 1979). While it was shown that the specific activity, i.e., the catalytic state of the enzyme, can change, the results have been equivocal. One major problem in these studies was insufficient sensitivity of the methodology; only microgram quantities could be measured. As a result, subtle changes in enzyme mass per unit mass of liver could not be detected.

We have been systematically developing radioimmunoassays (Mazzotta & Veneziale, 1980; Johnson & Veneziale, 1980; Hansen & Veneziale, 1980; C. M. Veneziale, J. C. Donofrio, and M. Y. Mazzotta, unpublished experiments) for multiple carbohydrate metabolizing enzymes of the rabbit. The sensitivity of the radioimmunoassays has allowed us to measure nanogram changes in the intracellular enzyme concentration. Our methodology coupled with assayable activity measurements has permitted critical evaluation of changes in the specific activity of the enzyme. In this paper we report on the development of a radioimmunoassay for rabbit L-type pyruvate kinase which has been applied to livers from rabbits subjected to numerous hormonal states and starvation. Liver pyruvate kinase refers to the L-type isozyme and muscle pyruvate kinase to the M-type isozyme.

Materials and Methods

Purification and Assay of Liver Pyruvate Kinase. New Zealand white rabbits weighing 3000 g that had been maintained on a balanced 50–60% carbohydrate diet (Purina Checkers Rabbit Chow) were used.

Livers (350 g tissue wet weight) which had been frozen in liquid nitrogen were homogenized in 3 volumes of buffer containing 10 mM sodium phosphate, pH 7.5, 10 mM β-mercaptoethanol, 1 mM EDTA, 0.25 M sucrose, 2 mM $MgSO_4$, and 0.2 mM fructose bisphosphate (FDP). The homogenate was centrifuged and the supernatant adjusted to pH 5.0 as described by Ljungström et al. (1974). Solid $(NH_4)_2SO_4$ was added to 35% saturation; after 1 h the material was centrifuged for 15 min at 15000*g*. The supernatant was then brought to 45% saturation with solid $(NH_4)_2SO_4$. After centrifugation the precipitate was dissolved in a minimal volume of buffer (10 mM sodium phosphate, pH 6.0, 10 mM β-mercaptoethanol, 2 mM $MgSO_4$, and 0.2 mM FDP) and dialyzed overnight against 30 volumes of the same buffer. The dialysate was mixed batchwise with 200 mL of phosphocellulose equilibrated in the same buffer. After 2 h the phosphocellulose was poured into a column (2.5 × 60 cm) and eluted with 500 mL of the equilibrating buffer. Next the column was eluted with 500 mL of equilibration buffer 33 mM in sodium phosphate. Finally 75 mM sodium phosphate equilibration buffer containing 0.5 mM phosphoenolpyruvate (PEP) was used to elute the enzyme. Fractions containing

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enzyme activity were pooled and brought to 50% saturation with $(NH_4)_2SO_4$. After 4 h the mixture was spun for 30 min at 15000*g*. The pellet was reconstituted and dialyzed against Blue Sepharose pH 6.8 buffer: 10% glycerol (v/v), 20 mM Tris-HCl, pH 6.8, 2 mM $MgSO_4$, 10 mM β-mercaptoethanol, and 1 mM EDTA. The enzyme was applied to a Blue Sepharose CL-6B column (2.5 × 10 cm) and eluted as described by Riou et al. (1978). Fractions containing enzyme activity which eluted in the KCl–FDP gradient were pooled and brought to 50% saturation with $(NH_4)_2SO_4$. Pooled enzyme was centrifuged and reconstituted in buffer containing 50 mM sodium phosphate, pH 7.5, and 10% glycerol (v/v). This was applied to a Model 6000A Waters high-pressure liquid chromatograph (Waters Associates, Milford, MD) with two ^{125}I protein columns connected in series. The column was eluted with 50 mM sodium phosphate, pH 7.5, and 10% glycerol (v/v) at 1.0 mL/min. Fractions containing enzyme activity were pooled, and enzyme was precipitated by addition of $(NH_4)_2SO_4$ to 50% saturation. The precipitate was dissolved in 50 mM sodium phosphate buffer, pH 7.5, containing 10% glycerol (v/v), 2 mM $MgSO_4$, 10 mM β-mercaptoethanol, 1 mM EDTA, and 0.2 mM FDP and stored at 4 °C or frozen.

Liver pyruvate kinase activity was determined spectrophotometrically by using a lactate dehydrogenase coupled reaction system that followed the decrease in NADH absorbance at 340 nm. The reaction cuvette contained 50 mM Tris-HCl, pH 7.6, 2.5 mM ADP, 7.5 mM $MgSO_4$, 75 mM KCl, 0.31 mM NADH, 5 units of LDH, and 1.5 mM FDP. Phosphoenolpyruvate (11.3 mM) was added to start the reaction after blank values for NADH oxidation were determined. The reaction was carried out at 25 °C in a final volume of 2.0 mL. For the purpose of assaying type-L pyruvate kinase activity in liver extracts, an aliquot form the 114000*g* supernatant of a 20% homogenate was brought to 45% saturation with $(NH_4)_2SO_4$ following a procedure used by others (Tanaka et al., 1967; Kohl & Cottam, 1976). We validated that this procedure precipitated only L-type pyruvate kinase by inhibition studies with specific γ-globulin. After centrifugation, the pellet was reconstituted in 0.05 M sodium phosphate, pH 7.6, and assayed for activity.

Antibody to Liver Pyruvate Kinase. Antibody to purified enzyme was raised in goats as previously described (Mazzotta & Veneziale, 1980). Methods for evaluation of antibody specificity and tissue enzyme inhibition studies have also been described elsewhere (Johnson & Veneziale, 1980).

Radioimmunoassay. Purified liver pyruvate kinase was iodinated by the method of Bolton–Hunter (Greenwood et al., 1963) as described in a previous publication (Mazzotta & Veneziale, 1980). After gel filtration in Bio-Gel P-300, the peak fractions of ^{125}I-labeled pyruvate kinase which had mobility identical with that of unlabeled enzyme were pooled and used as labeled antigen for the radioimmunoassay. A 15000-cpm amount of ^{125}I-labeled pyruvate kinase was incubated with enough γ-globulin to precipitate 40% of the label (when no competing unlabeled enzyme was present), increasing amounts of unlabeled enzyme, and 0.1 M phosphate buffer, pH 7.5, containing 1% ovalbumin. This provided the standard curve for quantitation of unknown liver tissue pyruvate kinase concentration.

Rabbits were subjected to various dietary and hormonal states prior to analysis. Diabetes was induced by intravenous injection of alloxan monohydrate (100 mg/1000 g body weight). Seventy-two hours later animals were sacrificed or insulin therapy was begun. Insulin was given subcutaneously once each day and 1 h before sacrifice on the final day.

Table I: Purification of Rabbit Liver Pyruvate Kinase[a]

step	vol (mL)	protein (mg)	act. (units)	sp act. (units/mg)	recovery (%)	purifn (x-fold)
crude homogenate supernatant	1050	42662	6938	0.16		1
pH supernatant	1500	25290	4895	0.19	71	1.2
35–45% $(NH_4)_2SO_4$ ppt	250	6428	2580	0.40	37	2.5
phosphocellulose column	34	284	2500	8.80	36	55
Blue Sepharose CL-6B column	2	3.14	340	108	5	675
LC[b]	2	2.41	270	112	3.9	700

[a] Protein was determined by the Hartree (1972) modification of the Lowry et al. (1951) method. Activity was determined by assay as described under Materials and Methods. [b] LC = high-pressure liquid chromatography.

Animals received 2 units on the first day and 1 unit each day thereafter. The rabbits receiving glucagon were given one subcutaneous injection (1 mg) every 6 h with the final injection 1 h before sacrifice. Thyroxine-treated animals received daily intramuscular injections of 20 μg. Triamcinolone (4 mg) was given intramuscularly once each day. Unless fasted, all rabbits were maintained on Purina Checkers Chow which was 50–60% carbohydrate. At the time of sacrifice, animals were anesthetized with ether and bled through severed carotid and jugular vessels. Livers were excised, washed in ice-cold saline, blotted dry, weighed, and homogenized in 4 volumes of 0.25 M sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, and 10 mM Tris, pH 7.6.

The 114000*g* supernatant fractions obtained from the homogenates were diluted 1:25, 1:50, 1:100, and 1:250. Liver pyruvate kinase concentrations were determined in 100-μL aliquots of each dilution in duplicate. The assay mixtures were exactly as described above for the standard curve except that standard unlabeled enzyme was omitted. Specific details of our radioimmunoassay methods and data analysis have been described elsewhere in detail (Mazzotta & Veneziale, 1980; Johnson & Veneziale, 1980; Hansen & Veneziale, 1980).

Results

Table I shows the purification procedure for rabbit liver pyruvate kinase (PK). The enzyme was purified 700-fold to constant specific activity. Enzyme stability was a considerable problem during the final steps of the purification, and 10% glycerol seemed to provide maximum stabilization. The large loss in enzyme activity during the Blue Sepharose CL-6B column procedure is partially a reflection of enzyme inactivation and not actual loss of enzyme protein. The purified enzyme was homogeneous on anionic gel electrophoresis and sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis (Veneziale & Deering, 1976) even when gels were greatly overloaded. The subunit molecular weight as determined by the method of Weber & Osborn (1969) was 59000.

Kinetic analysis of the purified enzyme demonstrated a sixfold allosteric activation by FDP (0.15 mM). The K_m for PEP is 0.27 mM in the presence of FDP. This correlates closely with a K_m value of 0.2 mM reported by others (Irving & Williams, 1973). In the absence of FDP, the K_m for PEP is 1.6 mM.

The specificity of the antibody preparation was evaluated by several methods. Figure 1 shows an inhibition study using purified enzyme. Five milligrams of antiliver pyruvate kinase could completely inhibit purified liver pyruvate kinase. Nonimmune γ-globulin controls did not inhibit enzyme ac-

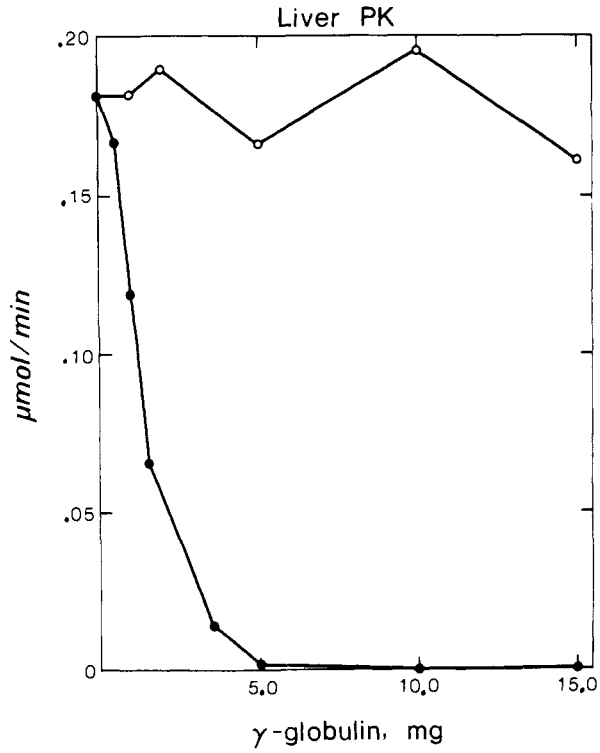

FIGURE 1: Inhibition study of purified liver pyruvate kinase with specific γ-globulin (●) and nonimmune γ-globulin (○). 100 μg of purified liver pyruvate kinase was incubated for 24 h at 4 °C in a final volume of 1.0 mL with the corresponding amount of γ-globulin buffered at pH 7.5. The incubations were then spun at 5000*g* for 30 min. 300 μL of the supernatants was assayed for enzyme activity.

tivity. Antiliver PK γ-globulin exhibited no cross-reactivity with purified muscle PK, and antimuscle PK γ-globulin exhibited no specificity for purified liver PK. Thus, the two isozymes represent immunologically distinct proteins. This was further validated by our demonstration that purified muscle PK did not compete in the liver radioimmunoassay. Furthermore, when purified preparations of rabbit liver fructose-1,6-bisphosphatase, muscle phosphofructokinase, and liver α-L-glycerolphosphate dehydrogenase and commercially obtained crude preparations of these enzymes were added, no competition was observed in the liver pyruvate kinase radioimmunoassay. Addition of a skeletal muscle extract (which contains no L-type pyruvate kinase) to the standard curve did not produce competition, indicating that nonspecific interference by tissue proteins does not occur in the radioimmunoassay. By Ouchterlony double-diffusion studies the specific antibody preparation gave a single line of identity between purified enzyme and liver extract.

Forty to sixty percent of the measurable pyruvate kinase activity in liver extracts was inhibited by antiliver pyruvate kinase γ-globulin. Of the remaining pyruvate kinase activity in liver extracts, all but 5% was inhibited by the antimuscle pyruvate kinase γ-globulin. Thus, rabbit liver contains both L- and M-type pyruvate kinases and a minor amount of the third isozyme. This agrees with the electrophoretic studies of Osterman & Fritz (1973).

Figure 2 is a composite of experiments done for the development of the radioimmunoassay. ^{125}I-Labeled enzyme was applied to a Bio-Gel P-300 column (1.1 × 100 cm), and 1.0-mL fractions were collected. Fractions 24–30 were pooled for use as ^{125}I-labeled enzyme for the radioimmunoassay. The ^{125}I-labeled enzyme had identical mobility to the native unlabeled subunit when examined by $NaDodSO_4$ gel electrophoresis (upper insert). The ability of our antiliver pyruvate

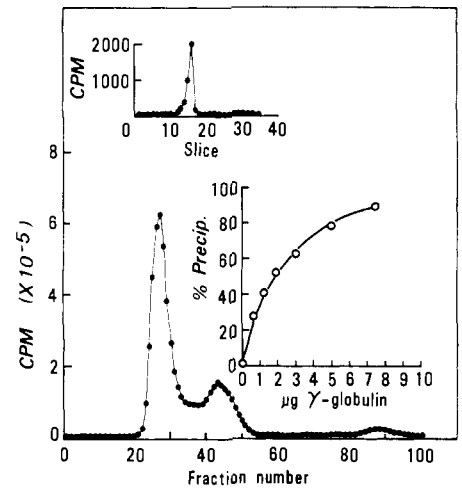

FIGURE 2: Bio-Gel P-300 column elution profile of ^{125}I-labeled liver pyruvate kinase. 20 μCi of [^{125}I]enzyme labeled as described under Materials and Methods was applied to the column. The column was eluted with 0.05 M phosphate buffer, pH 7.5, and 1.0-mL fractions were collected. Upper insert: a 50-μL aliquot of pooled fractions 24–30 was electrophoresed in our $NaDodSO_4$ gel system. Gels were frozen and sliced into 2.5-mm sections and counted for radioactivity. Lower insert: precipitation of ^{125}I-labeled enzyme. 15 000 cpm of ^{125}I-labeled enzyme, 0.1 M phosphate buffer (pH 7.5)–1% ovalbumin, and increasing amounts of γ-globulin as shown were incubated at 4°C. After 24 h, second-stage antigoat γ-globulin was added and incubation was continued for 4 h more. Percent precipitation was determined by counting the total cpm, centrifuging, and, after removal of the supernatant, counting the remaining pellet for radioactivity. Background was only 2 to 3%.

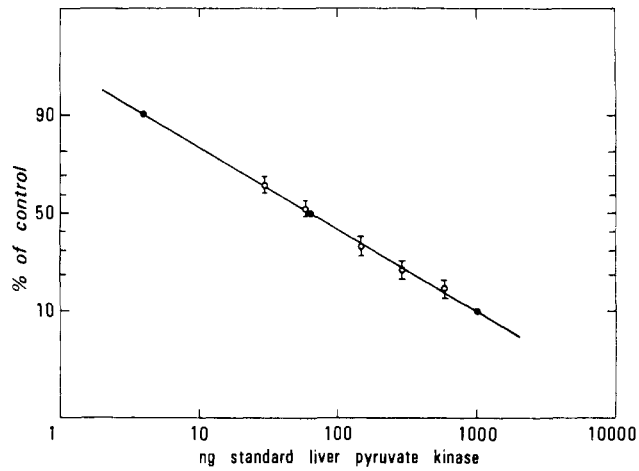

FIGURE 3: Radioimmunoassay standard curve for analysis of tissue enzyme concentration. The mean (○) and standard deviation (error bars) of 16 values for each amount of unlabeled enzyme are shown. Linear regression analysis provided the fitted line (●). See Materials and Methods for specific details.

kinase γ-globulin preparation to precipitate labeled enzyme was tested. A maximum of 87% was precipitated by 7.5 μg of γ-globulin (lower insert). A 1.25–1.75-μg amount of γ-globulin was needed to precipitate 40–50% of the labeled enzyme.

Figure 3 is a composite standard curve generated from eight different standard curves. The radioimmunoassay could accurately detect 10–1000 ng of liver pyruvate kinase. The reproducibility between tissue dilutions was ±5% over the entire range of the standard curve.

The results of the radioimmunoassay for liver pyruvate kinase are shown in Table II. Rabbits were subjected to the conditions shown (see Materials and Methods for details), and unless fasted all rabbits were maintained on the Purina

Table II: Liver Pyruvate Kinase[a]

condition	animal wt (g)	liver wt (g)	conc (μM)	nmol/liver	units/g of liver	units/liver	units/nmol of PK	N
chow fed	1326 ± 230	53 ± 17	1.28 ± 0.24	70 ± 34	16.1 ± 2.9	887 ± 436	12.7 ± 1.0	9
96-h fasted	1286 ± 114	34 ± 4	0.99 ± 0.11	33 ± 5	10.2 ± 1.8	343 ± 80	10.2 ± 1.7	5
		$p < 0.05$	$p < 0.05$	$p < 0.005$	$p < 0.005$	$p < 0.05$	$p < 0.005$	
diabetic untreated	1031 ± 132	42 ± 9	1.06 ± 0.30	46 ± 21	10.0 ± 2.4	431 ± 182	9.6 ± 1.5	8
	$p < 0.05$				$p < 0.005$	$p < 0.05$	$p < 0.005$	
4-day insulin-treated diabetic	1535 ± 199	66 ± 5	1.04 ± 0.10	68 ± 6	11.9 ± 2.3	784 ± 189	11.5 ± 1.8	4
glucagon treated	1223 ± 193	68 ± 21	0.99 ± 0.22	68 ± 25	10.1 ± 2.4	698 ± 280	10.2 ± 1.0	6
			$p < 0.05$		$p < 0.005$		$p < 0.005$	
fasted glucagon treated	1502 ± 122	42 ± 4	1.06 ± 0.31	45 ± 13	8.8 ± 2.5	372 ± 101	8.4 ± 1.4	6
					$p < 0.005$	$p < 0.05$	$p < 0.005$	
3-day triamcinolone treated	1222 ± 170	58 ± 21	0.96 ± 0.11	57 ± 26	7.0 ± 0.9	409 ± 165	7.4 ± 1.3	5
			$p < 0.05$		$p < 0.005$	$p < 0.05$	$p < 0.005$	
thyroxine treated	1458 ± 90	74 ± 9	1.44 ± 0.41	110 ± 44	20.2 ± 6.7	1532 ± 651	14.0 ± 3.5	6
		$p < 0.05$				$p < 0.05$		

[a] Rabbits were subjected to the conditions indicated; N represents the number of rabbits in each group. Concentration (μmol/1000 g tissue wet weight) was determined by radioimmunoassay. Units per gram of liver represents assayable activity measured in the extracts. Units per nanomole of PK represents the enzyme specific activity. Total liver enzyme (nanomoles per liver) and total liver activity (units per liver) were obtained by multipying liver weight by the concentration and units per gram of liver, respectively. All statistical analysis was based on comparison with chow-fed rabbits by using the Student's t test. All data are presented as the mean ± standard deviation.

Checkers Chow feed which served for the control untreated group. Animals weighing between 1100 and 1600 g body weight prior to treatment were used in these studies. Livers were routinely weighed to permit calculations of total organ quantities. All radioimmunoassays and activity measurements were performed on fresh extracts.

The greatest contrast to the control group were the 96-h fasted rabbits. Concentration and enzyme activity were significantly decreased. Also, liver weight was decreased which results in a large reduction in total nanomoles of enzyme per liver and total units of activity per liver. The change in enzyme concentration did not parallel the reduction in activity, and therefore specific activity was decreased.

In the diabetic animal the enzyme activity decreased, which coupled with no change in enzyme concentration resulted in a lowered specific activity of 9.6 units/nmol. After 4 days of insulin treatment the specific activity was 11.5 units/nmol, and after 8 days (not shown) the specific activity was 13.8 units/nmol. Thus, the enzyme was restored to the catalytic state of the control by 4 days and maintained by continued insulin injections.

Glucagon given to a chow-fed rabbit decreased the concentration slightly but more significantly decreased the activity per gram of liver and the specific activity of the enzyme. When the glucagon-treated animal was simultaneously fasted, both the total liver activity and total nanomoles of enzyme per liver decreased, largely due to differences in liver size.

The effects of a single dose of glucagon given 1 h prior to sacrifice were also studied (data not shown). In three of six rabbits there was a decrease in both enzyme activity (9.9 ± 1.2 units/g of liver) and specific activity (10.3 ± 0.9 units/nmol of PK), indicating a rapid action on the enzyme.

A surprising observation was the effect of the glucocorticoid triamcinolone on the enzyme. This hormone produced the greatest decrease in units per gram of liver and alteration of specific activity (12.7 units/nmol of PK in the control to 7.4 units/nmol of PK in a 3-day treated rabbit). The effect of triamcinolone at 24 h was also studied; the specific activity was decreased to 8.2 units/nmol of pK. Because the triamcinolone preparation was a suspension, absorption into the venous circulation from an intramuscular injection presents a problem for time intervals shorter than 24 h. Dexamethasone, which is a soluble glucocorticoid, was given 1 h prior to sacrifice. In four of five animals studied the units per gram of liver was significantly decreased, giving a specific activity of 10.7 ± 1.1 units/nmol of PK ($p < 0.05$). Thus, the inhibition of liver pyruvate kinase by glucocorticoids occurred rapidly and continued during prolonged hormone administration.

Thyroxine treatment, in contrast to all of the other hormones studied, resulted in small increases in the enzyme parameters. However, because the standard deviation for the data was large, the statistical significance of the results is minimal. Only in total activity per liver and in liver weight were the differences large enough to be significant. Nonetheless, the concentration, activity, and specific activity all tended to increase as a result of thyroxine treatment.

Discussion

Application of the radioimmunoassay and activity assays to livers from animals maintained on diets identical with those from which purified enzyme was isolated revealed a major difference in the specific activity of purified enzyme vs. tissue enzyme. The specific activity of purified enzyme was 26.4 units/nmol while that of control animals was 12.7 units/nmol. This discrepancy which has been observed by others working with rat liver pyruvate kinase (Kohl & Cottam, 1976; Hopkirk & Bloxham, 1979) could be the result of an inaccurate radioimmunoassay. The amount of enzyme detected could be anomalously high due to nonspecific competition. Therefore, we carefully examined a number of other enzyme and proteins for nonspecific competition in the radioimmunoassay. We were unable to demonstrate nonspecific competition in the radioimmunoassay, which provides support for its accuracy validity. An alternative explanation is that inactive enzyme which is lost during the purification procedure is detected in the radioimmunoassay of tissue extract. Another possibility is that unidentified components of tissue extracts could lower assayable activities and, therefore, give lower specific activity values for the tissue enzyme.

The reduction in enzyme activity in the diabetic and the return to control values after insulin therapy have been previously demonstrated (Weber et al., 1965; Tanaka et al., 1967). The concentration of enzyme in the diabetic liver was not significantly different from the control value, and insulin administration to the diabetic did not increase the enzyme concentration. The increase in nanomoles per liver after insulin treatment is the result of increased liver weight. Therefore, it appears that insulin is promoting a general anabolic effect rather than the induction of enzyme in preexisting cells. The

specific effect of insulin on liver pyruvate kinase was to restore the catalytic state of the enzyme to normal.

The inhibitory effect of glucagon on a number of liver pyruvate kinase kinetic parameters coincident with an increase in [^{32}P]phosphate incorporation into the enzyme has been well documented (Ljungström et al., 1974; Riou et al., 1978; Ishibashi & Cottam, 1978). We observed a marked reduction in enzymatic activity, agreeing with these and other earlier studies (Veneziale et al., 1976). Our current work goes beyond previous studies in that we directly demonstrated that glucagon decreases the specific activity of the enzyme.

Fasting caused a significant loss in liver weight which resulted in a twofold decrease in total nanomoles of enzyme. Kohl & Cottam (1976) reported a threefold decrease in total liver enzyme in the fasted rat. Paradoxically, however, they reported an increase in the specific activity of the enzyme. This disagrees with our specific activity data; we observed a significant decrease. Possibly the discrepancy was due to a failure of their methodology to measure the total amount of liver enzyme accurately (e.g., both active and less active or inactive enzyme molecules). The concentration of the enzyme also decreased after fasting, suggesting an alteration in pyruvate kinase synthesis or degradation.

The administration of triamcinolone produced marked inhibition of liver pyruvate kinase activity in contrast to the results of Weber et al. (1965), who were unable to demonstrate any effects of triamcinolone in the rat. Diamant et al. (1975) did observe an inhibition of liver pyruvate kinase activity on a per milligram of protein basis in the female rat. Because we have applied a radioimmunoassay, our experiments examined glucocorticoid effects in more detail than these earlier studies. In addition to a decrease in activity, we demonstrated a great decrease in the specific activity of the enzyme, thereby establishing a major feature of the triamcinolone action. If one examines the time course of glucocorticoid inhibition of the enzyme, at 1 h postinjection the specific activity was reduced to 10 units/nmol, after 24 h of treatment to 8.2 units/nmol, and after 3 days to 7.4 units/nmol.

The mechanism(s) mediating these hormonal effects are directly altering the enzyme's catalytic state. A phosphorylation–dephosphorylation mechanism is totally consistent with our data. This would explain the rapid inhibition by glucagon and glucocorticoids. However, a second mechanism may also be operating. This is suggested by data from the glucagon-treated fasted rabbits vs. the glucagon-treated rabbits and the 3-day triamcinolone-treated rabbits because the specific activity under these conditions was reduced significantly ($p < 0.05$) below that value observed after glucagon or fasting alone. A proteolytic event, as suggested by Hall et al. (1979), which further inactivates an already phosphorylated enzyme molecule is a possibility.

The increase in enzyme parameters induced by thyroxine, contrasted with the other results, is not completely defined by the data presented in this paper. It would appear that activation of the enzyme occurs, but as both concentration and total nanomoles per liver increased slightly, induction of new catalytically more active enzyme is a possibility. More experiments will be needed to fully explain the effects of thyroxine.

The data presented in this paper demonstrate the complex nature of the mechanisms involved in regulation of liver pyruvate kinase. Fasting, diabetes, insulin, glucagon, and triamcinolone all had direct effects on the catalytic state of the enzyme. In addition, changes in total liver enzyme mass were shown to occur and are also important in determining total liver capacity to carry out glycolysis or gluconeogenesis. Changes in total organ enzyme mass often reflected changes in organ weight. What might be the nature of an increase in the amount of organ enzyme, as, for example, under the influence of a hormone? If the liver weight and the number of hepatic parenchymal cells remain constant, then an increase in enzyme mass could be considered a true induction phenomenon. If liver weight increases due to an increase in the number of liver cells, then any increase in pyruvate kinase could be due to the de novo synthesis of enzyme obligated to the appearance of new cells. Thus, the hormone would presumably have influenced growth and development of hepatic parenchymal cells rather than the genetic expression of pyruvate kinase in preexisting cells. To distinguish these situations and to investigate if hormonal induction (or repression) is possible, we have begun to apply our radioimmunoassay method to known numbers of cells in culture.

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