Hepatic Enzyme Activities in Streptozotocin-Diabetic Rats Before and After Insulin Treatment

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SUMMARY

Streptozotocin causes hyperglycemia and hypoinsulinemia in rats. Striking elevations in hepatic phosphoenolpyruvate carboxykinase, pyruvate carboxylase, hexosediphosphatase and glucose-6-phosphate, and dramatic decreases in glucokinase, pyruvate kinase and 6-phosphogluconate dehydrogenase are observed in these diabetic animals. Daily treatment of insulin in diabetic rats brings about increase in liver weight, glycogen and soluble protein contents, and in the levels of glucokinase, pyruvate kinase, 6-phosphogluconate dehydrogenase and lactate dehydrogenase. In terms of unit/gm. liver, the activities of PEP carboxykinase, glucose-6-phosphatase and hexosediphosphatase are depressed by insulin injection. However, a multiphasic pattern is observed in the changes of these gluconeogenic enzymes following insulin treatments when their activities are expressed as units/100 gm. body weight or unit/mg. protein. Insulin withdrawal causes immediate rise in gluconeogenic enzymes, particularly in the extramitochondrial activity of pyruvate carboxylase, and decreases in glucose-catabolizing enzymes which proceed at different rates. DIABETES *20*:71-77, February, 1971.

Both alloxan and streptozotocin induce diabetes in animals by their β-cytotoxic actions.[1] It has been well documented that, in alloxan-diabetic rats, the impaired homeostatic control of blood glucose is often accompanied by an anomaly in hepatic enzyme levels which can be ameliorated by insulin administration.[2,3] Little is known, however, about the levels of hepatic enzymes in streptozotocin-induced diabetic rats. The purpose of this study is to determine if these diabetic rats also have aberrant levels of hepatic gluconeogenic and glucose-catabolizing enzymes. Microsomal glucose-6-phosphatase and the activities of phosphoenolpyruvate (PEP) carboxykinase, pyruvate carboxylase, hexosediphosphatase, glucokinase, pyruvate kinase, 6-phosphogluconate dehydrogenase and lactate dehydrogenase present in the cytosol fraction were measured. In addition, the response of these enzymes toward daily administration of insulin in the diabetic rats is also reported.

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MATERIALS AND METHODS

Animals: Fasted, male Upj:TUC(SD)spf rats, weighing about 150 gm., were injected intravenously with a single dose of 65 mg./kg. of streptozotocin, freshly prepared by dissolving in saline (adjusted to pH 4.5 with 0.05 M citric acid). Control animals were given the same amount of saline. The animals were allowed food and water ad libitum following injection, and were used for studies at least eight weeks later. Each group consisted of six animals. Insulin was injected I.P. in a dosage of five units of protamine-Zn insulin per 100 gm. rat weight. All studies were initiated at eight o'clock in the morning.

Preparation of liver microsomal and supernatant fractions: Before the animals were decapitated, 2 ml. of blood was collected through orbital sinus for the determination of blood glucose and plasma insulin. The livers were quickly removed, rinsed in ice-cold 0.15 M KCl (adjusted to pH 7.4 with 0.02 M $KHCO_3$), blotted on filter paper and weighed in a tared beaker containing 5.0 ml. ice-cold 0.15 M KCl. Enough buffer was then added to make the final volume nine times the weight of liver. Liver was cut into small pieces and homogenized with nine strokes at 600 r.p.m. in a glass homogenizer with a Teflon pestle. The mixture was centrifuged at 8,000 $\times$ g for ten minutes and supernatant was further sedimented in a Spinco rotor 40 at 36,000 r.p.m. for seventy minutes. The 100,000 $\times$ g supernatant was collected, carefully avoiding the lipid layer, and assayed immediately for hexosediphosphatase, PEP carboxykinase, pyruvate carboxylase and glucokinase. A portion of the supernatant was quickly frozen in a dry ice-acetone bath and used to assay pyruvate kinase, 6-phosphogluconate dehydrogenase and lactate dehydrog-

TABLE 1

Comparison in various properties between control and streptozotocin-diabetic rats

Constituents		Control	Streptozotocin-diabetic	P Values
Animal weight	gm.	322.8 ± 13.4	222.8 ± 16.7	<0.001
Blood sugar	mg. per 100 ml.	96.3 ± 3.0	433 ± 53.1	<0.001
Plasma insulin	μU./ml.	109.5 ± 47	<5	
$\frac{\text{Liver wt.}}{\text{Body wt.}} \times 100$		4.18 ± 0.17	5.06 ± 0.19	<0.001
Liver soluble protein	mg./gm.	83.22 ± 4.95	107.2 ± 5.32	<0.001
Liver microsomal protein	mg./gm.	21.27 ± 1.63	31.66 ± 2.15	<0.001
Liver glycogen	mg./gm.	31.9 ± 5.5	14.7 ± 2.1	<0.001

Values represent the means of six animals ± standard deviation.

enase on the following day.

The microsome pellets were rinsed twice with ice-cold 0.1 M Tris-Cl, pH 7.6, 0.005 M $MgCl_2$ and suspended in the same buffer. It was transferred to a glass homogenizer and crushed with a Teflon pestle by hand. The final volume of microsome suspension was one-fifth of the original volume of crude homogenate. The microsomal fraction was used immediately to assay glucose-6-phosphatase activity.

Assay procedure: The following enzymes were measured by methods as indicated in the references: hexosediphosphatase,[4] PEP carboxykinase,[5] glucokinase,[2] pyruvate kinase,[6] 6-phosphogluconate dehydrogenase,[7] and lactate dehydrogenase.[4] Glucose-6-phosphatase was assayed according to Nordlie and Snoke[8] at pH 6.1. Pyruvate carboxylase was assayed with a modified method of Wagle.[9] In the reaction mixture, acetyl CoA was substituted by the addition of 1 μmole CoA, 10 μmole acetyl phosphate and 1 unit of phosphotransacetylase. PEP carboxykinase, pyruvate carboxylase, hexosediphosphatase and glucose-6-phosphatase were assayed at 37° C. following three minutes pre-incubation at the same temperature. Other enzymes were measured at room temperature.

Spectrophotometric assays were carried out in a Beckmann DB Spectrophotometer. Radioactivity was measured in Bray solution[10] in a Packard Tricarb Scintillation Spectrometer. Quenching was corrected by channel-ratio method.[11] Protein determination was performed according to Lowry et al.[12] with BSA as standards. Blood glucose was measured on an AutoAnalyzer by the method of Hoffman.[13] Plasma insulin was determined by immunoassay with bovine insulin as standards.[14] Liver glycogen was precipitated from 30 per cent KOH extract[15] and determined with the phenol-sulfuric acid method.[16]

Substrates, coenzymes and enzymes used in assays were purchased from Boehringer-Mannheim, New York, New York, or Sigma, St. Louis, Missouri. $NaH\text{-}^{14}CO_3$ (0.1 μC/μm) was obtained from New England Nuclear, Boston, Massachusetts, and subsequently diluted with nonradioactive material to the specific activity indicated. Protamine-Zn insulin was purchased from Eli Lilly, Indianapolis, Indiana.

TABLE 2

Hepatic enzyme levels of control and streptozotocin-diabetic rats

	μmole/min./100 gm. body weight		
	Control	Streptozotocin-diabetic	P Values
PEP carboxykinase	9.06 ± 1.19	26.5 ± 3.3	<0.001
Pyruvate carboxylase	0.082 ± 0.011	0.28 ± 0.147	<0.01
Hexosediphosphatase	44.4 ± 4.4	75.72 ± 7.25	<0.001
Microsomal glucose-6-phosphatase	28.3 ± 3.3	98.6 ± 12.4	<0.001
Glucokinase	1.87 ± 0.35	0.91 ± 0.28	<0.001
Pyruvate kinase	62.0 ± 11.7	18.0 ± 1.75	<0.001
6-P-gluconate dehydrogenase	11.7 ± 0.6	8.26 ± 0.82	<0.001
Lactate dehydrogenase	604 ± 81	746 ± 85	<0.02

Values represent the means of six animals ± standard deviation.
Enzyme assays are described in MATERIAL AND METHODS.

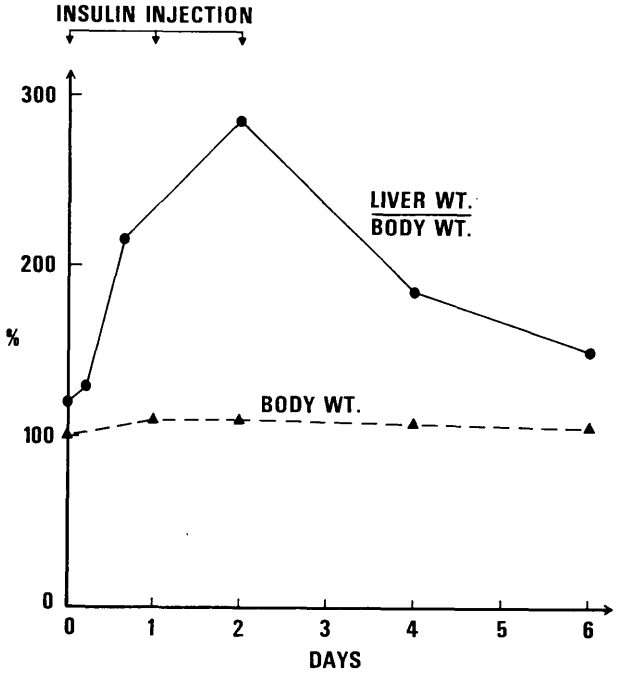

FIG. 1. Effect of insulin on liver and body weights in streptozotocin-diabetic rats. The ratio of liver weight to body weight (●—●) was calculated on the basis of % change from that observed in control rats (0.042 ± 0.0017, 100%). Body weight (▲—▲) was represented as % change from initial body weight in each group before the first injection of insulin.

RESULTS

Comparison between streptozotocin-diabetic and control rats: At the time of eight weeks after streptozotocin injection the diabetic rats experienced severe hyperglycemia and hypoinsulinemia, and gained little weight during the interval (table 1). In addition, they developed ketonuria and cataract. The diabetic rats also had more hepatic proteins in both soluble and microsomal fractions, higher ratio of liver to body weight, and less liver glycogen than the control rats.

Table 2 summarizes the levels of various hepatic enzymes, expressed in unit per 100 gm. body weight, in both diabetic and control rats. The activities of PEP carboxykinase, pyruvate carboxylase, hexosediphosphatase and glucose-6-phosphatase were greatly elevated in streptozotocin-treated rats. Conversely, the activities of glucokinase, pyruvate kinase and 6-phosphogluconate dehydrogenase were greatly reduced in the diabetic rats. Lactate dehydrogenase was slightly enhanced in these animals.

When the activities of these enzymes were calculated on the basis of unit per mg. protein or unit per gm. liver, the diabetic rats also showed elevated levels of gluconeogenic enzymes and depressed activities of glucose-catabolizing enzymes. In the case of lactate dehydrogenase, discrepancies among choices of definition of enzyme activity were observed, however. The diabetic rats had more hepatic lactate dehydrogenase per 100 gm. body weight (745 ± 85 to 604 ± 81, $P < 0.02$), normal amount per gm. liver (147 ± 12 to 145 ± 19), and subnormal level in terms of unit per mg. hepatic soluble proteins (1.37 ± 0.14 to 1.73 ± 0.18 unit/mg. soluble protein, $P < 0.01$).

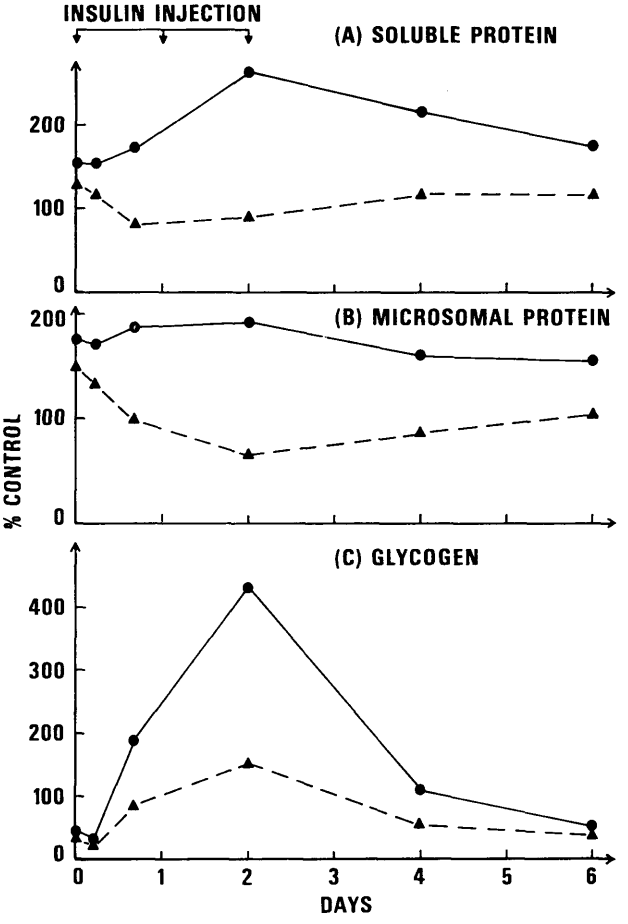

FIG. 2. Effect of insulin on liver compositions in streptozotocin-diabetic rats. Depicted points represent % change from control rats. Circles (●—●) indicate values obtained from concentration in mg. per 100 gm. body weight. Triangles (▲—▲) indicate values obtained from concentration in mg. per gm. liver.
(A). Soluble proteins (100,000 × g supernatant). Control rats had 348 ± 18 mg. per 100 gm. body weight and 83.2 ± 4.9 mg. per gm. liver.
(B). Microsomal proteins (8,000 × g to 100,000 × g sediments). Control rats had 88.9 ± 6.6 mg. per 100 gm. body weight and 21.3 ± 1.6 mg. per gm. liver.
(C). Glycogen. Control rats had 669 ± 161 mg. per 100 gm. body weight and 31.9 ± 5.5 mg. per gm. liver.

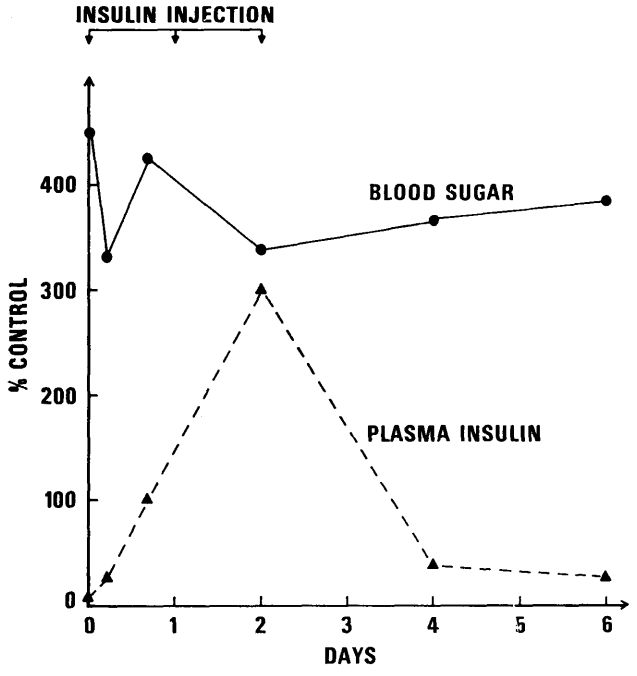

FIG. 3. Effect of insulin on blood sugar and plasma insulin levels in streptozotocin-diabetic rats. Depicted points represent per cent change from control rats. Control rats had 96.3 ± 3.0 mg. per 100 ml. blood glucose (●—●) and 109.5 ± 47 μunit/ml. plasma insulin (▲—▲).

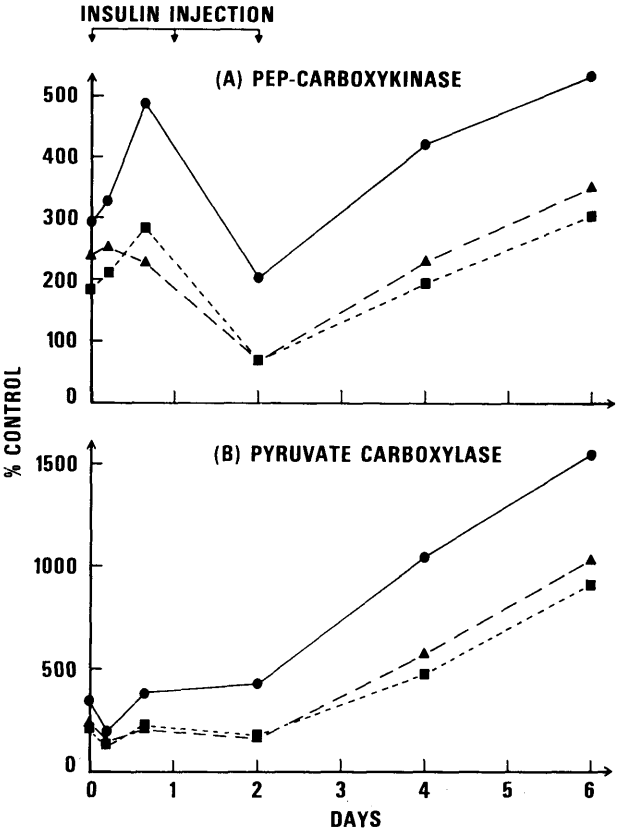

FIG. 4. Effect of insulin on hepatic PEP carboxykinase and pyruvate carboxylase in streptozotocin-diabetic rats. Depicted points represent per cent change from control rats. Circles (●—●) indicate values obtained from activity in unit per 100 gm. body weight. Triangles (▲—▲) indicate values obtained from activity in unit per gm. liver weight. Squares (■—■) indicate values obtained from activity in unit per mg. proteins. Assays are described in "Materials and Methods." Enzyme unit is defined as μmole C-14-O_2 fixed per minute at 37° C.

(A). PEP carboxykinase. Control rats had activities of 9.06 ± 1.19 units per 100 gm. body weight, 2.17 ± 0.28 units per gm. liver and 26.2 ± 4.1 μunits per mg. soluble protein.

(B). Pyruvate carboxylate. Control rats had activities of 82 ± 11 μunits per 100 gm. body weight, 19.7 ± 3.1 munits per gm. liver and 0.24 ± 0.035 munits per mg. soluble protein.

Effect of insulin on streptozotocin-diabetic rats: Daily injection of 5 unit/100 gm. of insulin in the diabetic rats brought about a slight gain in body weight and a tremendous increase in liver size which decreased rapidly upon cessation of insulin injection (figure 1). The increase in liver weight could be attributed mostly to glycogen deposition (figure 2-C), and, to lesser extent, augmentation in the amount of soluble protein (figure 2-A). Hepatic microsomal protein increased only slightly after insulin injection (figure 2-B).

Five hours after insulin administration, a significant drop in blood glucose was observed, and then followed by a rebound eleven hours afterwards (figure 3). A cumulative effect of successive injections of insulin was suggested by increasing level of plasma insulin (figure 3).

Effect of insulin on hepatic gluconeogenic enzymes: When enzyme activity was calculated on the basis of unit per 100 gm. body weight or mg. hepatic soluble proteins, the injection of insulin caused an initial increase in PEP carboxykinase level, a subsequent decline, and, finally, a rebound after withdrawal (figure 4-A). If the activity was expressed as unit per gm. liver, however, the initial increase in PEP carboxykinase was no longer observed, and yet, the subsequent decline and rebound were still in full evidence. In the case of pyruvate carboxylase, no matter what means of calculation was used, its activity present in the cytosol fraction showed only marginal changes during insulin administration and increased significantly after withdrawal (figure 4-B).

The activities per gram liver of both microsomal glucose-6-phosphatase and hexosediphosphatase decreased following insulin injection, and returned to the initial

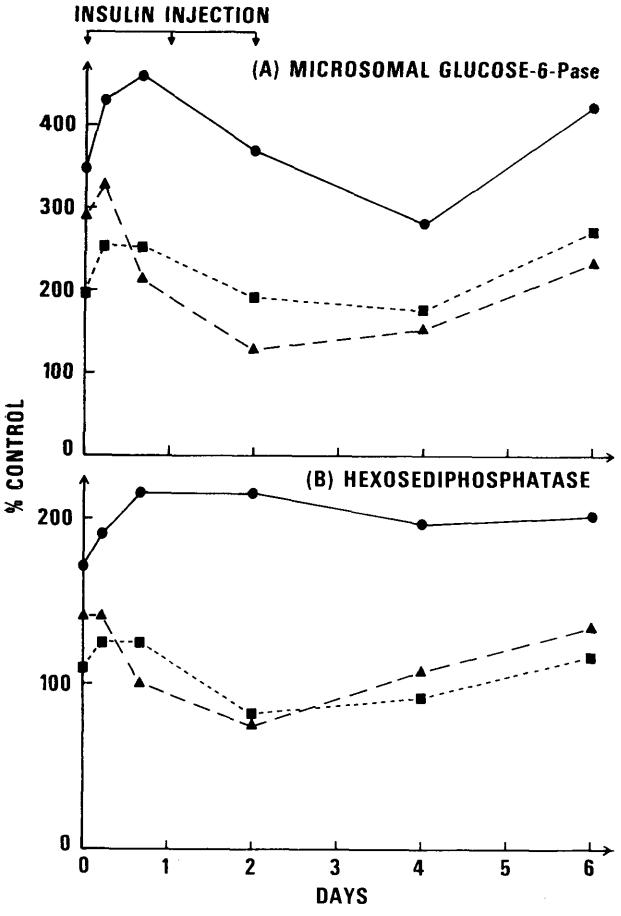

FIG. 5. Effect of insulin on hepatic glucose-6-phosphatase and hexosediphosphatase in streptozotocin-diabetic rats. See legend to figure 4. Enzyme unit is defined as μmole inorganic phosphate released per minute at 37° C.
(A). Microsomal glucose-6-phosphatase. Control rats had activities of 28.3 ± 3.3 units per 100 gm. body weight, 6.77 ± 0.76 units per gm. liver and 0.32 ± 0.02 units per mg. microsomal proteins.
(B). Hexosediphosphatase. Control rats had activities of 44.4 ± 4.4 units per 100 gm. body weight, 10.6 ± 0.7 units per gm. liver and 0.13 ± 0.01 units per mg. soluble proteins.

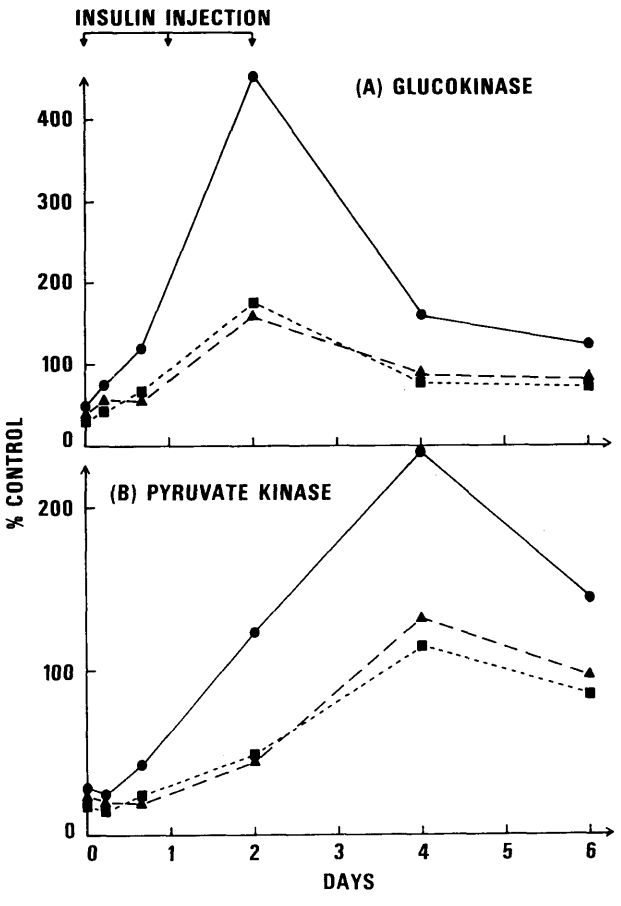

FIG. 6. Effect of insulin on hepatic glucokinase and pyruvate kinase in streptozotocin-diabetic rats. See legend to figure 4. Enzyme unit is defined as μmole NAD turnover per minute at room temperature.
(A). Glucokinase. Control rats had activities of 1.87 ± 0.35 units per 100 gm. body weight, 0.45 ± 0.08 units per gm. liver and 5.35 ± 0.78 munits per mg. soluble proteins.
(B). Pyruvate kinase. Control rats had activities of 62.0 ± 11.7 units per 100 gm. body weight, 14.8 ± 2.7 units per gm. liver, and 0.18 ± 0.03 units per mg. soluble proteins.

levels upon withdrawal (figure 5). On the other hand, in terms of unit per mg. hepatic proteins, these phosphatases changed only moderately in response to insulin administration. Furthermore, insulin treatment caused a slight elevation of hexosediphosphatase with respect to unit per 100 gm. body weight.

Effect of insulin on hepatic glucokinase, pyruvate kinase, 6-phosphogluconate dehydrogenase and lactate dehydrogenase: Hepatic glucokinase activity increased after insulin administration and its level followed closely with plasma insulin concentration (figure 6-A and 3). Insulin injection also brought on a slow elevation in pyruvate kinase activity, which began to decrease two days after withdrawal of insulin (figure 6-B). A similar but more prolonged effect of insulin on 6-phosphogluconate dehydrogenase was also observed and shown in figure 7. There was no discrepancy in the stimulatory effect of insulin on the levels of these enzymes among choices of definition of enzyme activity. On the other hand, the level of lactate dehydrogenase was significantly elevated by insulin administration only in terms of unit per 100 gm. body weight (figure 8).

DISCUSSION

As in alloxan diabetes,[2,3,17] streptozotocin-induced diabetic rats have elevated levels of hepatic PEP carbox-

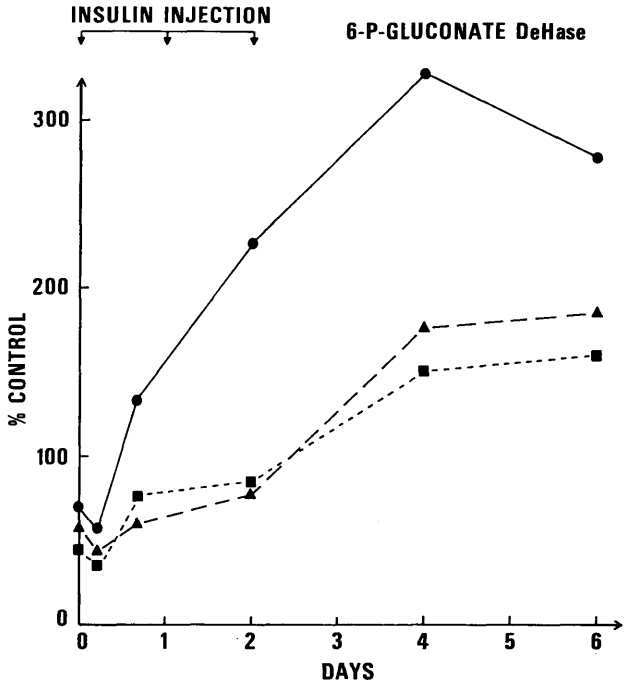

FIG. 7. Effect of insulin on hepatic 6-phosphogluconate dehydrogenase in streptozotocin-diabetic rats. See legend to figure 4. Enzyme unit is defined as μmole NADP reduced per minute at room temperature. Control rats had activities of 11.7 ± 0.6 units per 100 gm. body weight, 2.8 ± 0.1 units per gm. liver and 33.9 ± 2.2 munits per mg. soluble proteins.

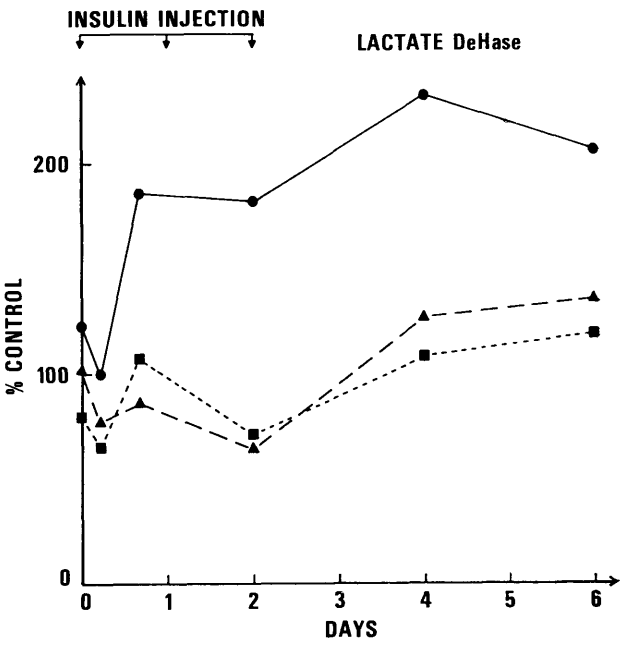

FIG. 8. Effect of insulin on hepatic lactate dehydrogenase in streptozotocin-diabetic rats. See legend to figure 4. Enzyme unit is defined as μmole NADH oxidized per minute at room temperature. Control rats had activities of 604 ± 81 units per 100 gm. body weight, 145 ± 19 units per gm. liver and 1.73 ± 0.18 units per mg. soluble proteins.

ykinase, pyruvate carboxylase, hexosediphosphatase and glucose-6-phosphatase, and reduced quantity of glucokinase, pyruvate kinase and 6-phosphogluconate dehydrogenase. It is generally held that the functional activity of a given metabolic pathway is reflected by the levels of enzymes regulating that pathway. Therefore, it appears that hyperglycemia in streptozotocin-treated rats arises from an accelerated rate of gluconeogenesis and depressed rates of glycolysis and pentose phosphate shunt.

The effect of insulin on reduction of PEP carboxykinase, hexosediphosphatase and glucose-6-phosphatase levels is evident only in terms of unit per gm. liver, which appears to result from an offsetting increase in liver weight. In view of the fact that insulin treatment causes only marginal effect on body weight, it would be more meaningful to interpret the results in terms of unit per 100 gm. body weight. It is interesting to note that insulin administration causes an initial rise and a subsequent decline in PEP carboxykinase and glucose-6-phosphatase levels, and that withdrawal of insulin effects a rebound in the activity of these enzymes. This suggests that the control of these gluconeogenic enzymes by insulin involves a complex mechanism and the suppressive action of insulin may be secondary rather than direct. The effect of insulin on hexosediphosphatase is only marginal.

The extramitochondrial activity of pyruvate carboxylase does not appear to be directly affected by the presence of insulin. However, insulin withdrawal brings about a precipitous ascent in the activity of this enzyme in cytosol fraction. It has been shown that the majority of pyruvate carboxylase activity is located in mitochondria in rat liver.[18] The observation thus supports the notion that an immediate effect of insulin withdrawal is to shift the location of a portion of this enzyme from mitochondria to cytosol.[19] Alternatively, insulin withdrawal may cause changes in mitochondria membrane which becomes more permeable and allows pyruvate carboxylase to leak through during fractionation.[19]

Our results on the levels of glucokinase and pyruvate kinase following insulin administration are in keeping with those reported in alloxan-diabetic rats by Sharma et al.[2] and Weber et al.[17] respectively. In addition, insulin also increases the activity of 6-phosphogluconate dehydrogenase in streptozotocin-diabetic rats. The fact that the insulin-dependent increases of these

glucose-catabolizing enzymes do not proceed at the same rate supports the notion that the control of these enzymes by insulin involves several independent mechanisms.[20]

In conclusion, certain metabolic abnormalities in streptozotocin-induced diabetic rats can be, at least in part, attributed to changes in hepatic enzyme activities which are, in turn, results of insulin deficiency. The regulation of the levels of key enzymes in gluconeogenesis, glycolysis and pentose phosphate shunt appears to involve several independent mechanisms.

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