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Research Article

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The Rapid Changes of Hepatic Glycolytic Enzymes and Fructose-1,6-Diphosphatase Activities after Intravenous Glucagon in Humans

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ABSTRACT Glucagon (0.04–0.09 mg/kg/min) was given intravenously for either 2 or 3 min to eight patients with fasting-induced hypoglycemia. One child had hepatic phosphorylase deficiency, two children had glucose-6-phosphatase deficiency, two children had debrancher enzyme (amylo-1,6-glucosidase) deficiency, and two children and one adult had decreased hepatic fructose-1,6-diphosphatase (FDPase) activity. Liver biopsy specimens were obtained before and immediately after the glucagon infusion. The glucagon caused a significant increase in the activity of FDPase (from 50 ± 10.0 to 72 ± 11.7 nmol/mg protein/min) and a significant decrease in the activities of phosphofructokinase (PFK) (from 92 ± 6.1 to 41 ± 8.1 nmol/mg protein/min) and pyruvate kinase (PK) (from 309 ± 39.4 to 165 ± 23.9 nmol/mg protein/min). The glucagon infusion also caused a significant increase in hepatic cyclic AMP concentrations (from 41 ± 2.6 to 233 ± 35.6 pmol/mg protein). Two patients with debrancher enzyme deficiency who had biopsy specimens taken 5 min after the glucagon infusion had persistence of enzyme and cyclic AMP changes for at least 5 min. One child with glucose-6-phosphatase deficiency was given intravenous glucose (150 mg/kg/min) for a period of 5 min after the glucagon infusion and biopsy. The plasma insulin concentration increased from 8 to 152 μ U/ml and blood glucose increased from 72 to 204 mg/100 ml. A third

liver biopsy specimen was obtained immediately after the glucose infusion and showed that the glucagon-induced effects on PFK and FDPase were completely reversed. The glucagon infusion caused an increase in hepatic cyclic AMP concentration from 38 to 431 pmol/mg protein but the glucose infusion caused only a slight decrease in hepatic cyclic AMP concentration (from 431 to 384 pmol/mg protein), which did not appear to be sufficient to account for the changes in enzyme activities. Hepatic glucose-6-phosphatase and fructose-1,6-diphosphate aldolase activities were not altered by either the glucagon or the glucose infusion in any patients. Cyclic AMP (0.05 mmol/kg) was injected into the portal vein of adult rats and caused enzyme changes similar to those seen with glucagon administration in humans. Our findings suggest that rapid changes in the activities of PFK, PK, and FDPase are important in the regulation of hepatic glycolysis and gluconeogenesis, respectively, in humans and that cyclic AMP may mediate the glucagon- but probably not the glucose-insulin-induced changes in enzyme activities.

INTRODUCTION

Glucagon and insulin play a central role in the rapid regulation of hepatic gluconeogenesis and glycolysis, but the exact mechanism of their inverse relationship in this regulatory action is not known. The respective rates of glycolysis and gluconeogenesis appear to have more than one mechanism of regulation (2), and changes in enzyme activities are one possible mechanism of control (3). Other workers (4–8) have shown that insulin and glucocorticoids may affect enzyme activities by altering the rate of protein synthesis of certain glycolytic and gluconeogenic enzymes, but since protein synthesis ordinarily is expected to take several hours this

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TABLE I
Clinical Data of Patients

Patient	Age	Sex	Enzyme deficiency	Weight	Height	Biopsy technique*	Anesthesia or analgesia	Reason for surgery
	yr			kg	cm			
1	10	M	hepatic phosphorylase	25	122	open	Na pentothal	P-C shunt†
2	10	F	glucose-6-phosphatase	27	124	open	Na pentothal	P-C shunt
3	7	F	glucose-6-phosphatase	16	104	closed	Ketamine HCl	diagnostic
4	2	F	fructose diphosphatase	11	82	closed	Ketamine HCl	diagnostic
5	2	F	fructose diphosphatase	11	84	closed	Ketamine HCl	diagnostic
6	25	F	fructose diphosphatase	52	160	closed	Meperidine HCl	diagnostic
7	5	F	amylo-1,6-glucosidase	16	98	open	Na pentothal	P-C shunt
8	3	M	amylo-1,6-glucosidase	14	88	open	Na pentothal	P-C shunt

* No complications occurred with any of the biopsies.

† P-C shunt, portacaval venous anastomosis created for the treatment of glycogen storage disease (10).

alteration would not account for the hormone-induced changes in blood glucose that occur within a matter of minutes after an insulin or glucagon infusion. Data on the short-term effects of hormones on these enzymes are largely unexplained.

In rats we have recently demonstrated that within minutes the intravenous infusion of glucagon or insulin significantly altered the activities of certain enzymes unique to the glycolytic and gluconeogenic pathways (9). Glucagon and insulin were reciprocal in their effects on the enzyme activities. Furthermore, the glucagon-induced changes were completely reversed within 3-min after an insulin infusion. Hepatic cyclic AMP concentrations increased 3- to 4-fold within 2 min after glucagon but were not significantly affected by intravenous insulin. After glucagon, insulin did decrease cyclic AMP levels slightly but not enough to account for the alteration of enzyme activities (9).

This communication extends our observations to man. We studied the acute effects of intravenous glucagon on human hepatic enzyme activities and cyclic AMP concentrations in eight patients with various types of hepatic enzyme deficiencies.

METHODS

Patients. All studies were carried out after obtaining informed consent.¹ The patients were seven children and one adult who were being treated for fasting-induced hypoglycemia. Table I lists the individual patients' pertinent physical characteristics and the specific enzymatic defect for which they were receiving treatment or being studied. All patients who had open biopsies were undergoing elective surgery for a portacaval venous anastomosis as treatment for glycogen storage disease (10). Since some degree of hypoglycemia usually occurred with fasting, all patients

¹ Studies were carried out on a formal protocol basis with approval of the Human Research Committee and in accordance with the principles of the Declaration of Helsinki.

except the 25-yr-old woman received intravenous glucose (25–35 mg/min) for ½–1 h before liver biopsy. Patients 2, 7, and 8 had been receiving total parenteral nutrition for 2 wk and were receiving it at the time of surgery. The open biopsies in the children were performed during sodium pentothal anesthesia and the closed biopsies with ketamine HCl (3 mg/kg i.m.). The adult patient was biopsied after a 7-h fast and 20 min after meperidine HCl (50 mg i.v.) analgesia.

Biopsies. Percutaneous liver biopsies were performed with the Menghini biopsy needle (1.4 mm). When we were testing for changes in enzyme activities, an initial biopsy specimen was obtained, an intravenous infusion of glucagon (0.04 to 0.09 mg/kg/min) was given with an infusion pump for either 2 or 3 min, and a second biopsy was obtained immediately. Two patients had the second biopsy specimen taken 5 min after the infusion (patients 7 and 8). One patient with glucose-6-phosphatase deficiency was given an infusion of glucose (150 mg/kg/min) for 5 min after the glucagon administration, and a third biopsy specimen was taken. Liver biopsy specimens were separated into appropriate portions for assay of cyclic AMP (frozen within 10 s in liquid nitrogen), histology, and measurement of the various enzyme activities.

Assays. Phosphofructokinase (PFK)² assays were performed on fresh tissues. Liver obtained for enzyme analysis was weighed and assayed fresh or frozen (–70°C) immediately and assayed later. Except for PFK, freezing and storage up to 1 mo caused no significant changes in the enzyme activities. The fresh or frozen tissue was placed in a Kontes-Duall homogenizing tube (Kontes Glass Co., Vineland, N. J.) with 20 vol of ice-cold buffer (pH 7.5), which contained 20 mM Tris, 120 mM KCl, 5 mM MgSO₄, and 0.1 mM EDTA (disodium salt). The samples were homogenized with a Teflon pestle, placed in 2-ml Beckman cellulose nitrate tubes and centrifuged at 104,000 *g* for 60 min in a Beckman L-2 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 4°C. The lipid layer was aspirated and discarded and the clear supernate was decanted without disturbing the pellet.

Enzymes were assayed spectrophotometrically (340 nm)

² **Abbreviations used in this paper:** FDPA, fructose-1,6-diphosphate aldolase; FDPase, fructose-1,6-diphosphatase; GK, glucokinase; PEP-CK, phosphoenolpyruvate carboxykinase; PFK, phosphofructokinase; PK, pyruvate kinase.

TABLE II
Effect of Glucagon on

Patient	GK			PFK			PK		
	Before‡	After	Change	Before	After	Change	Before	After	Change
	nmol/mg protein/min		%	nmol/mg protein/min		%	nmol/mg protein/min		%
Normal adults* (n = 10)	29 ± 3.4						116 ± 30.3		
1	47	28	-41	—	—		444	129	-57
2	—	—		—	—		324	226	-31
3	—	—		109	58	-47	510	291	-43
4	—	—		91	50	-45	194	80	-59
5	—	—		81	21	-74	264	114	-57
6	—	—		86	36	-58	262	186	-30
7	79	51	-36	—	—		229	150	-35
8	85	56	-35	—	—		244	140	-43
Mean ± SE	70 ± 11.8	45 ± 8.6		92 ± 6.1	41 ± 8.1		309 ± 39.4	165 ± 23.9	
P		<0.025			<0.001			<0.01	

* Patients undergoing abdominal surgery and without liver disease. Mean and standard error of mean are given.

‡ "Before" indicates that biopsy was performed just before the glucagon infusion, which lasted 3 min in all patients except patient 3. "After" indicates that the second biopsy was obtained immediately after the glucagon infusion except for patients 7 and 8, who had the second biopsy 5 min after the glucagon infusion was completed.

§ Blood glucose determinations were made at 0 (Before), 2, 5, and 10 min after the glucagon infusion was begun (After).

|| Immediately after the glucagon infusion, intravenous glucose (150 mg/kg/min) was infused for 5 min.

at room temperature on the supernatant fraction as described previously (11, 12). The assays were performed simultaneously, under identical conditions for all samples from the same patient. PFK was assayed by the method of Lea and Walker (13) with minor changes. Briefly, the reaction mixture contained 4.0 μ mol imidazole buffer, pH 7.4, 5 μ mol MgCl₂, 0.1 μ mol NADH, 2.4 μ mol glucose-6-phosphate, 1.1 μ mol ATP, 2.1 μ mol AMP, 200 μ mol KCl, 0.3 μ mol KCN, 500 μ g aldolase, 50 μ g alpha-glycerol phosphate dehydrogenase, 50 μ g phosphoglucoseisomerase, 20 μ g triose phosphate isomerase, and 20 μ l of the 104,000 g supernate. The reaction was initiated by adding glucose-6-phosphate and the final volume was 0.985 ml. The pH of the ATP and AMP stock solutions was adjusted to 7.0 and stored at -20°C. Pyruvate kinase (PK) (14), fructose-1,6-diphosphatase (FDPase) (15), fructose-1,6-diphosphate aldolase (FDPA) (16), glucokinase (GK) (17), and glucose-6-phosphatase (18) activities were assayed as described previously (11, 12) with 20 μ l of the 104,000 g supernate. Enzyme activities are expressed as nanomoles of substrate metabolized per minute per milligram of protein. Cyclic AMP concentrations were measured by the method of Gilman (19). The final cyclic AMP concentrations in the standard curve ranged from 4 to 18 pmol. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (20). Blood glucose determinations were performed by the glucose oxidase method and insulin measured by radioimmunoassay. Analysis of data was made by Student's *t* test (21).

Diets. The patients were studied on a metabolic ward while eating a constant 1,200-1,800-Cal liquid diet to maintain a constant weight. The caloric proportions were 25% dextrimaltose, 25% glucose, 20% casein (Casec, Mead Johnson Laboratories, Div. of Mead Johnson & Co., Evansville, Ind.), and 30% corn oil. Standard amounts of

vitamins and minerals were added to the diet. This diet was given for 7 days before beginning the studies.

Animal studies. Six adult male Carworth rats (400-500 g), (Carworth Div., Becton, Dickinson & Co., New City, N. Y.) fed ad libitum on Purina chow (Ralston Purina Co., St. Louis, Mo.), were anesthetized with 50 mg/kg of pentobarbital intraperitoneally. A 21-gauge scalp vein needle was inserted into the portal vein and the tubing flushed with 1 ml of 0.9% saline. 4 min later (zero time) 30-40 mg of liver was removed. Cyclic AMP in one group of three rats and 5'AMP in another group of three rats was injected at a dose of 0.05 mmol/kg in 0.1 ml of 0.9% saline. Subsequent liver specimens were taken at 5, 10, and 15 min after the injection and the enzymes were assayed fresh.

RESULTS

Table II demonstrates the changes in enzyme activities, cyclic AMP, and blood glucose concentrations in the individual patients after intravenous glucagon. Although there were different initial values and different degrees of response to glucagon of the various enzyme activities and cyclic AMP concentrations, statistical differences were demonstrated between the before and after mean values. There was a significant decrease in the activities of GK from 70 ± 11.8 to 45 ± 8.6 nmol/mg protein/min ($P < 0.025$), of PFK from 92 ± 6.1 to 41 ± 8.1 nmol/mg protein/min ($P < 0.001$), and PK from 309 ± 39.4 to 165 ± 23.9 nmol/mg protein/min ($P < 0.01$). The activity of FDPase was significantly increased by glucagon from 50 ± 10.0 to 72 ± 11.7 nmol/mg protein/

Hepatic Enzyme Activities

FDPA		FDPase			CyclicAMP		Blood Glucose	
Before	After	Before	After	Change	Before	After	Before	After§
nmol/mg protein/min		nmol/mg protein/min		%	pmol/mg protein		mg/100 ml	
85±19.4		57±5.2			33±5.2			
129	106	76	97	+22	48	202	93	98, 110, 117
145	143	66	99	+33	47	302	98	96, 99, 101
174	182	52	85	+39	38	424	68	72, 148 , 180
137	130	16	26	+39	38	290	88	88, —, 118
117	112	20	46	+57	28	160	96	100, 124, 136
112	93	18	28	+6	44	106	87	90, 112, 115
117	116	78	98	+21	37	184	106	104, —, 118
108	106	79	98	+21	41	193	91	94, —, 105
130±7.7	123±9.9	50±10.0	72±11.7		40±2.3	233±35.6		
NS			<0.001		<0.001			

min ($P < 0.001$). The activity of FDPA was not significantly altered by glucagon. These changes in enzyme activities were accompanied by a marked increase in hepatic cyclic AMP concentrations from 40 ± 2.3 to 233 ± 35.6 pmol/mg protein ($P < 0.001$). Similar changes in the enzyme activities were seen whether the specimens were taken at 3 min or 5 min (patients 7 and 8) after the glucagon infusions were completed. These findings indicate that the glucagon-induced enzyme changes existed for at least 5 min after the glucagon infusion. Glucose-6-phosphatase activities (not shown in the table and measured in only two patients) did not change after the glucagon infusion.

In one patient with hepatic glucose-6-phosphatase deficiency, a 2-min glucagon infusion and biopsy was followed by an intravenous infusion of glucose at a dose of 150 mg/kg/min for 5 min. Fig. 1 shows that there was reversal of the glucagon effect on the enzyme activities, except for PK, by the glucose infusion. Blood insulin values are given in the lower portion of the figure, which shows a several-fold increase over that seen before the glucose infusion. The blood glucose values were 68, 72, and 204 mg/100 ml, which corresponded to the insulin levels of 8, 16, and 152 μ U/ml, respectively. Unlike the changes in enzyme activities, the glucose infusion was associated with only a minimal decrease in cyclic AMP concentrations.

Fig. 2 demonstrates the enzyme changes seen in rat liver tissue after a portal vein infusion of 0.05 mmol/kg

of cyclic AMP, and 5'AMP. Cyclic AMP caused enzyme changes similar to those seen after glucagon administration in man. There was no significant change in the glycolytic enzyme activities after 5'AMP infusion, but a significant though small decrease in the activity of FDPase from 49.1 ± 2.8 to 40.0 ± 2.7 nmol/mg protein/min ($P < 0.05$) occurred within 5 min after the infusion.

DISCUSSION

The long-term effects of insulin and glucocorticoids on the changes in certain enzymes involved in glycolysis and gluconeogenesis (4-8) as well as certain other hepatic enzymes (22, 23) have been reported previously. In our studies we have investigated the short-term effects of glucagon on three enzymes unique to glycolysis, PFK, PK, and GK, and one enzyme unique to gluconeogenesis, FDPase. PFK, PK, and GK are associated with essentially irreversible reactions and are generally thought to be a major means of controlling the rate of glycolysis. In the case of the gluconeogenetic pathway, pyruvate carboxylase and phosphoenolpyruvate carboxylase (PEP-CK) appear to be the primary rate-limiting enzymes, but under certain circumstances, FDPase may affect the overall rate of glucose synthesis (24). This phenomenon is of particular importance in the patients with hypoglycemia due to FDPase deficiency (25, 26).

In eight patients, we have shown that within 3-5 min, an intravenous infusion of glucagon caused a significant

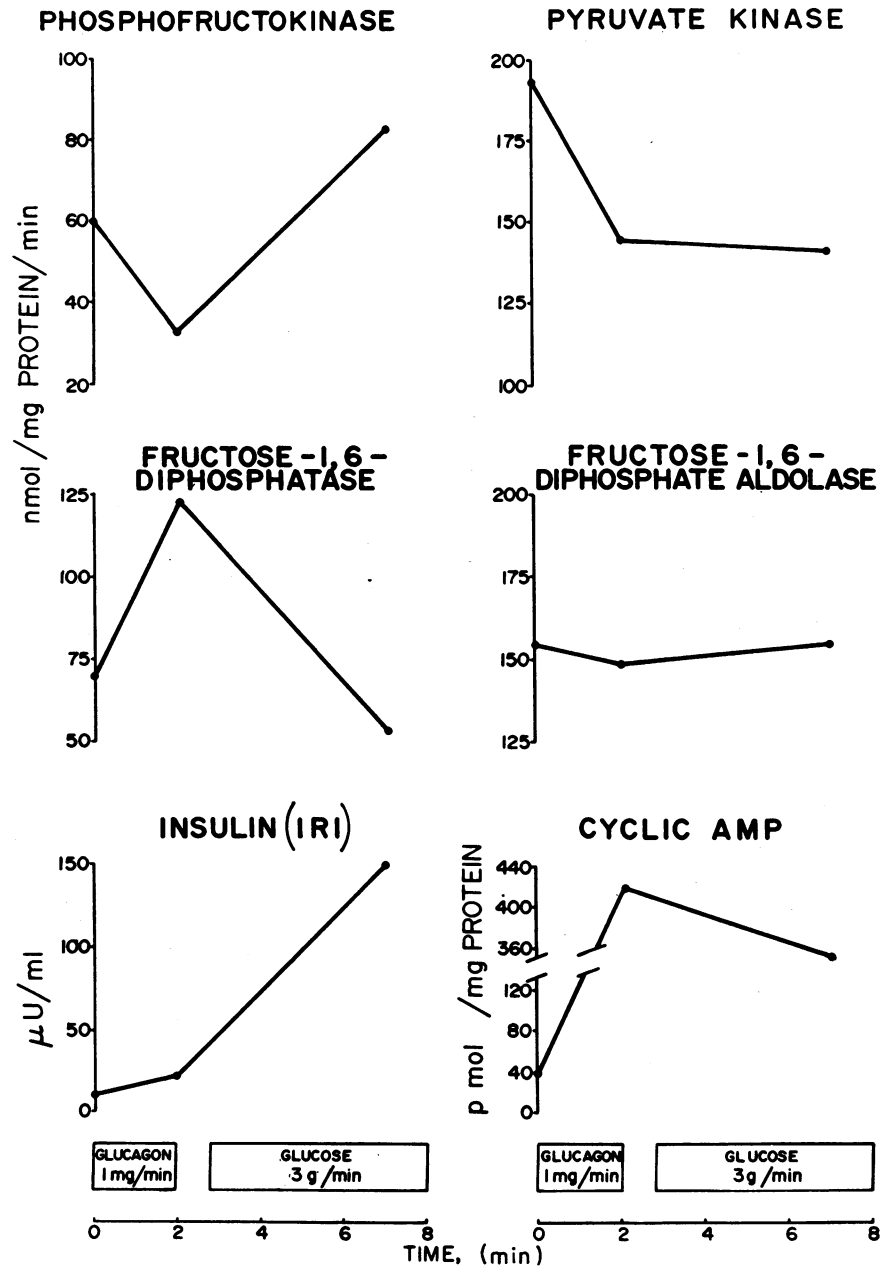


FIGURE 1 Effect of intravenous glucagon followed by intravenous glucose on hepatic enzyme activities, blood insulin (IRI) and cyclic AMP concentrations in one patient with hepatic glucose-6-phosphatase deficiency.

decrease in the activity of PK and a reciprocal increase in the activity of FDPase. PFK activity was measured in four patients and decreased significantly with the glucagon infusion. In three patients, there was a decrease in GK activity after the glucagon infusion. This enzyme was not measured in the remaining patients.

There were no glucagon-induced changes in the activity of the reversible enzyme FDPase. The rapid effects

of glucagon on PFK, PK, and FDPase were associated with a significant increase in hepatic cyclic AMP concentrations. In rat experiments, after an epinephrine infusion (0.5-4.0 μ g/min.) the increase in cyclic AMP concentrations preceded the enzyme changes by 30-60 s and neither effect was inhibited by puromycin or actinomycin-D (27). Identical studies with glucagon have given similar results (28). It is of interest that Jost,

Hsie, Hughes, and Ryan (23) and Exton and Park (29) have demonstrated that glucagon rapidly increased hepatic cyclic AMP concentrations in vivo in rats and in the isolated perfused rat liver, respectively.

Two patients showed that the glucagon-induced enzyme effect persisted for at least 5 min after the infusion was stopped. In another patient, the glucagon-induced effect on PFK and FDPase was reversed within 5 min by a glucose infusion that caused a significant increase in insulin concentrations. The glucose caused little change, however, in the cyclic AMP concentration. These findings are similar to those seen in rats infused with epinephrine (27) or glucagon (28) and then insulin except that in the animals, the glucagon-induced effect on PK was also reversed by the insulin infusion.

The changes in cyclic AMP concentrations do not in themselves prove that cyclic AMP is the mediator of the rapid enzyme changes seen after glucagon. However, the cyclic AMP-induced changes in rat liver enzymes do lend support to the concept that cyclic AMP is directly involved in the glucagon-induced alterations in enzyme activities and may therefore affect the rate of gluconeogenesis and glycolysis within a matter of minutes (30). The glucose-insulin effect on the changes in enzyme activities do not appear to be mediated by cyclic AMP since the small decrease in cyclic AMP concentrations did not seem sufficient to account for the enzyme changes caused by the glucose infusion in one patient and the epinephrine-insulin or glucagon-insulin infusions in rats. However, we cannot preclude the possibility that insulin alters the ratio between free and bound cyclic AMP so that, although the total cyclic AMP may not change, the active species of cyclic AMP may be diminished.

FDPase activity has been shown to be inhibited by 5'AMP in vitro (31, 32). This was the only enzyme significantly affected by the 5'AMP infusion in rats. There are conflicting reports about the role of FDPase in regulating gluconeogenesis and whether glucagon exerts any action at this point. Veneziale and Blair, Cook, and Lardy have presented data that indicate that glucagon causes an increase in the rate of glucose formation from either fructose (33) or xylitol and dihydroxyacetone (34) when the PEP-CK reaction is inhibited by quinolinate. However, other investigators have not supported these findings (35-39). Our studies, showing that glucagon rapidly increased the activities of FDPase, are more consistent with the observations of Veneziale (33) and Blair et al. (34). In view of the acute in vivo effects of glucagon on FDPase in the rat (9, 27, 28), the findings of Veneziale and Blair et al, the effect of glucagon on human hepatic enzymes presented in this paper, and the existence of the clinically significant condition, FDPase deficiency (25, 26), it

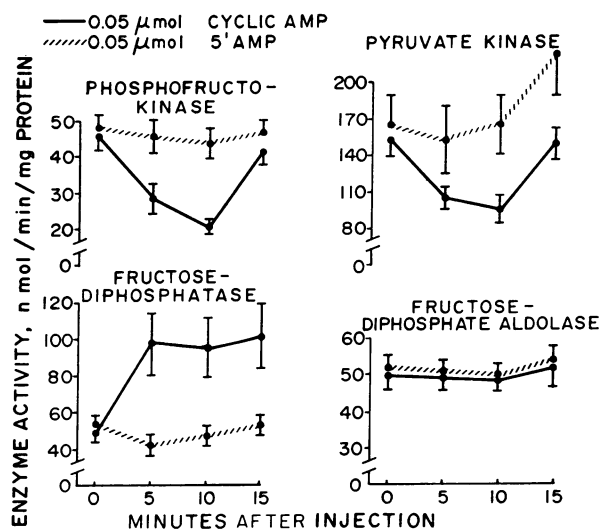


FIGURE 2 Rat hepatic enzyme activities after a portal vein infusion of 0.05 mmol/kg cyclic AMP (solid lines) and 0.02 mmol of 5'AMP (broken lines). The vertical lines through each point represent SEM.

seems reasonable to conclude that the FDPase reaction is an important point in the regulation of gluconeogenesis and that part of this regulatory control is mediated by glucagon. It may be that part of the difficulty in demonstrating an increase in the production of glucose from fructose in the presence of glucagon, was due to the fact that fructose, in substrate amounts, accumulates as fructose-1-phosphate and persists during the peak action of glucagon. This has been shown to be due to a trapping of inorganic phosphate as fructose-1-phosphate with a resultant increase in the activity of AMP deaminase that is inhibited by inorganic phosphate. This in turn causes the formation of IMP, which inhibits the action of fructose-1-phosphate aldolase (40-42). Thus, this would effectively prevent fructose from reaching the FDPase step.

FDPase, PFK, and PK are subject to modulation by a number of physiologically important substances such as 5'AMP (reversible by pyridoxal phosphate), fructose diphosphate, cystamine, and possibly unsaturated fatty acids for FDPase; ATP, ADP, AMP, cyclic AMP, Ca^{++} , fructose-6-phosphate and citrate for PFK; and phosphoenol pyruvate, fructose diphosphate, alanine, and histidine for PK (24). Perhaps the changes in enzyme activities that we observed are mediated through some or all of these substances. However, some of the modulating effects by these compounds are demonstrable only in vitro and there is no single species whose action can consistently account for all the enzyme changes. The finding that cyclic AMP can mimic the glucagon effects suggests that this compound may be a common

modulator for the rapid control of the enzymes in glycolysis and gluconeogenesis. Our findings as well as those of Venezia and Blair et al. suggest that the reported inhibitory effect of cyclic AMP on FDPase and its stimulatory effect on PFK in vitro (43, 44) may not occur under physiologic conditions.

The mechanism whereby these enzyme activities are altered by glucagon is not known. Since a major effect of glucagon is glucose mobilization from the liver to the blood, it is conceivable that in addition to the activation of liver phosphorylase, certain key enzymes in gluconeogenesis are activated by a similar mechanism. Therefore, a unitary hypothesis for the action of glucagon on hepatic release of glucose would involve the simultaneous activation of glycogenolysis and gluconeogenesis, and inactivation of glycogen formation and glycolysis. This mechanism of activation and inactivation of gluconeogenesis and glycolysis, respectively, could possibly involve cyclic AMP and protein kinase(s) in a manner analogous to phosphorylase activation in glycogenolysis and inactivation of glycogen synthetase in glycogen formation (45, 46). However, no data are presently available to suggest that any of the enzymes measured in this study are activated or inactivated by phosphorylation.

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