Rapid Reciprocal Changes in Rat Hepatic Glycolytic Enzyme and Fructose Diphosphatase Activities following Insulin and Glucagon Injection

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SUMMARY

Insulin and glucagon injected into the portal vein produced rapid reciprocal changes in the activities of certain hepatic glycolytic enzymes and fructose diphosphatase in the rat. Insulin produced a rapid increase in hepatic phosphofructokinase and pyruvate kinase activities and a decrease in fructose diphosphatase activity. Glucagon produced a rapid but reciprocal response in each of these enzyme activities. Fructose diphosphate aldolase activity was unaltered by either hormone.

The insulin effect on these enzymes was detected within 5 min following injection, was maximal by 10 min, and then gradually declined over the remaining 30 min of testing. The magnitude of the insulin effect was dependent on the amount of insulin injected. No effect was detected when 0.005 unit per kg (0.001 unit) was injected; phosphofructokinase and pyruvate kinase activities were slightly increased with 0.015 unit per kg (0.003 unit); phosphofructokinase, pyruvate kinase, and fructose diphosphatase activities were altered with 0.15 unit per kg (0.03 unit) and 1.5 units per kg (0.3 unit), with the higher dose giving a slightly greater effect. The insulin effect was not associated with a significant change in cyclic adenosine 3':5'-monophosphate concentrations.

The glucagon effect on the enzyme activities was preceded by a significant increase in cyclic adenosine 3':5'-monophosphate concentrations, which occurred within 30 s after hormone injection. A change in enzyme activities was found at 2 to 5 min and was maximal 5 to 10 min following glucagon injection. The hormone effect on enzyme activities and cyclic adenosine 3':5' monophosphate levels was dependent on the amount of glucagon injected, being maximal at doses of 150 to 300 μg.

Pretreatment of the rat with actinomycin D or puromycin did not alter the response of the enzymes to insulin and glucagon, indicating that de novo protein synthesis was not responsible for the change in enzyme activities. Glucagon injected 5 min following insulin reversed the insulin effect; insulin given 5 min after glucagon also partially reversed the glucagon effect on cyclic adenosine 3':5'-monophosphate and the enzyme activities.

Intravenous insulin produced rapid changes in the activities of renal cortical, skeletal muscle, and epididymal fat glycolytic enzymes and fructose diphosphatase in the rat. Insulin (0.15 unit per kg) produced rapid increases in pyruvate kinase and phosphofructokinase and rapid decrease in fructose diphosphatase activities in all tissues. Fructose diphosphate aldolase activity was unchanged following insulin infusion.

Intravenous glucagon (0.15 mg) produced rapid changes, reciprocal to those seen with insulin, in fructose diphosphatase activity in all tissues. Glucagon significantly decreased epididymal fat phosphofructokinase activities but did not alter the activity of this enzyme in the renal cortex and skeletal muscle. Glucagon significantly decreased renal cortical pyruvate kinase but had no effect in the epididymal fat and skeletal muscle. Fructose diphosphate aldolase activity was unchanged in all tissues following glucagon infusion.

Cyclic adenosine 3':5'-monophosphate infusion (0.05 mmole) produced significant changes in pyruvate kinase, phosphofructokinase, and fructose diphosphatase activities in all tissues which resembled those changes seen following glucagon infusion.

These data suggest that the glucagon responses seen in the four tissues studied are mediated by cyclic adenosine 3':5'-monophosphate.

The antagonistic effects of insulin and glucagon on hepatic carbohydrate metabolism are well established. These two hormones have opposing effects on glycogen synthesis and breakdown, glycolysis, and gluconeogenesis. The action of insulin and glucagon on glycogen metabolism seems established and involves the regulation of glycogen synthetase and phosphorylase by phosphorylation-dephosphorylation mechanisms (1-3). On
injection, tissue from the opposite kidney, epididymal fat pad, control samples was obtained from one kidney, one epididymal quick excised (within 30 a) for enzyme analysis. Tissue for the entire fat pad was removed and a portion of the tissue was NaCl solution and a control sample (epididymal fat, approximately 50 to 100 mg of muscle and renal cortex and 200 to 400 mg of skeletal muscle) or 10 volumes (for epididymal fat) of ice-cold buffer (pH 7.5) which consisted of 20 mM Tris-HCl, 120 mM KCl, 5 mM MgSO4, and 0.1 mM EDTA (disodium salt). The liver samples were kept on ice until all were obtained and then were homogenized with a Teflon pestle just prior to centrifugation. All other tissues were homogenized immediately. The homogenate was placed in 2 ml Beckman cellulose nitrate tubes and centrifuged at 104,000 X g for 60 min in a Beckman L-2 ultracentrifuge at 4°. The lipid layer was aspirated and discarded and the clear supernatant was then decanted without disturbing the pellet.

In separate experiments, cyclic AMP or 5'-AMP was injected into the portal vein at a dose of 0.05 nmole/kg in 0.1 ml of 0.9% NaCl solution. Tissue specimens were taken 5, 10, and 15 min following the injection and the enzymes were assayed fresh. The specificity for anesthesia and the intravenous procedure are identical with those described above.

The following enzymes were assayed spectrophotometrically at room temperature with the use of a Gilford recording spectrophotometer at 340 nm: pyruvate kinase (11), fructose 1,6-diphosphate aldolase (12), phosphofructokinase (13), and fructose 1,6-diphosphatase (14). The phosphofructokinase assay was modified so that the reaction mixture contained 40 pmole of imidazole (pH 7.4), 0.2 mumole of NADH, 2.3 mumoles of glucose 6-phosphate, 1.1 mumoles of ATP, 2.1 mumoles of AMP, 200 mumoles of KCl, 0.3 mumole of KCN, 5 mumoles of MgCl2, 500 pg of aldolase, 500 pg of triosephosphate isomerase, 20 pg of glucose 6-phosphate dehydrogenase, 20 pg of triosephosphate isomerase, and 20 pl of 104,000 X g supernatant. 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°. The pyruvate kinase assay was modified so that the reaction mixture contained 23 mumoles of triethanolamine (pH 7.0), 200 mumoles of KCl, 0.2 mumole of NADH, 8.9 mumoles of ADP, 0.7 mumole of phosphoenolpyruvate, 10 mumoles of MgCl2, 30 pg of lactic acid dehydrogenase, and 20 pl of the 104,000 X g supernatant. The reaction was initiated by adding phosphoenolpyruvate, and the final volume was 0.975 ml. The fructose diphosphate aldolase assay was modified so that the reaction mixture contained 66 mumoles of triethanolamine and 1.75 mumoles of fructose 1,6-diphosphate, 30 pg of a-glycerophosphate dehydrogenase, 12 pg of triosephosphate isomerase, and 20 pl of the 104,000 X g supernatant. The reaction was initiated by adding fructose 1,6-diphosphate, and the final volume was 0.925 ml. The fructose diphosphatase assay was identical with the method described by Racker (14), except that 3 pg of glucose 6-phosphate dehydrogenase and 35 pg of glucose 6-phosphate isomerase were used. The remaining assay mixture was identical: 100 mumoles of Tris (pH 8.8), 1 mumole of fructose 1,6-diphosphate, 1.6 mumoles of EDTA-Na2, 50 mumoles of MgCl2, 0.25 mumole of TPN, and 20 pl of the 104,000 X g supernatant, giving a final volume of 0.825 ml. Enzyme activity is expressed as micromoles of substrate metabolized per min per mg of protein or as determined by the method of Lowry et al. (15), using crystalline bovine albumin as a standard. Plasma glucose was determined in a Beckman automatic analyzer by the glucose oxidase method (16).

Cyclic AMP was measured by the method of Gilman (17). Cyclic AMP concentrations in the standard curve ranged from 4 to 18 pmole. Cyclic AMP was measured in the liver samples. Cyclic AMP was assayed in triplicate from each sample at each time point as previously described (8). Recoveries were monitored by adding approximately 6000 dpm of cyclic [PH]AMP to each sample prior to homogenization. All samples were assayed in triplicate. The protein precipitated by the trichloroacetic acid was dissolved in 1 ml of 1 N NaOH and determined by the method of Lowry et al. (15). Cyclic AMP concentration is expressed as piconoles per mg of trichloroacetic acid-precipitated protein.

Puromycin dihydrochloride (23 mg) was injected intraperi-

**MATERIALS AND METHODS**

Crystalline insulin and glucagon were obtained from Eli Lilly. Cyclic [PH]AMP (16.3 Ci per mmole) was obtained from Schwarz-Mann. Cyclic AMP, other nucleotides, enzymes, and sugars used in the enzyme assays, and puromycin dihydrochloride were obtained from Sigma Chemical Co. Actinomycin D was obtained from Calbiochem. Protein kinase (9) and protein kinase inhibitor (10) used in the cyclic AMP assay were prepared from fresh bovine skeletal muscle. The protein kinase purification was taken through the DEAE-cellulose chromatography step.

Male Carworth rats (150 to 250 g), housed in individual cages and fed Purina rat chow ad libitum (glucagon experiments) or fasted for approximately 16 hours (insulin experiments), were anesthetized with 50 mg per kg of pentobarbital intraperitoneally and their abdomens were opened. A 21-gauge scalp vein needle was inserted into the portal vein and blood was allowed to fill the tubing. The blood was flushed from the tubing with approximately 1 ml of sterile 0.9% NaCl solution. The hormone vehicle, 0.15 to 0.30 ml, was injected and flushed into the vein with 1 ml of sterile 0.9% NaCl solution; a control liver sample was excised 4 min later. Approximately 10 to 20 mg of liver were removed and immediately frozen (within 5 to 10 s) by immersion into liquid nitrogen for cyclic AMP determination. Approximately 50 mg of liver were then excised for enzyme analysis. All liver samples were taken randomly from different sites in the liver. No sample was taken contiguous to a previous sampling site. A small piece of dry cotton gauze was placed over the liver excision site for hemostasis. A gauze pad was placed over the exposed liver, and fed Purina rat chow ad lib&m (glucagon experiments) or 0.9% NaCl solution over a 5- to 10-s period. Liver samples were kept on ice until all were obtained and then were homogenized with a Teflon pestle just prior to centrifugation. All other tissues were homogenized immediately. The homogenate was placed in 2 ml Beckman cellulose nitrate tubes and centrifuged at 104,000 X g for 60 min in a Beckman L-2 ultracentrifuge at 4°. The lipid layer was aspirated and discarded.

In studies of epididymal fat, renal cortex, and skeletal muscle (rectus femoris), a 21-gauge scalp vein needle was inserted into the infantile yellow fat and blood was allowed to fill the tubing. The blood was flushed from the tubing with 0.3 ml of sterile 0.9% NaCl solution and a control sample of tissue (epididymal fat, renal cortex, or skeletal muscle) was excised 4 min later. Approximately 50 to 100 mg of muscle and renal cortex and 200 to 400 mg of epididymal fat were excised for immediate enzyme analysis. The entire fat pad was removed and a portion of the tissue was quickly tied at the appropriate time, and the entire kidney was removed. The kidney was bisected, the medullary tissue was dissected free, leaving mostly cortex, and the sample was homogenized within 60 to 90 s.

Liver and other tissues obtained for enzyme analysis were immediately weighed and placed in a Kontes-Dual 1 homogenizer with either homogenate buffer (pH 7.5) which consisted of 20 mM Tris-HCl, 120 mM KCl, 5 mM MgSO4, and 0.1 mM EDTA (disodium salt). The liver samples were kept on ice until all were obtained and then were homogenized with a Teflon pestle just prior to centrifugation. All other tissues were homogenized immediately. The homogenate was placed in 2 ml Beckman cellulose nitrate tubes and centrifuged at 104,000 X g for 60 min in a Beckman L-2 ultracentrifuge at 4°. The lipid layer was aspirated and discarded and the clear supernatant was then decanted without disturbing the pellet.

The abbreviations used are: cyclic AMP, cyclic adenosine 3':5'-monophosphate.

Glucagon vehicle, 1.6% glycerin and 0.2% phenol; insulin vehicle, sterile 0.9% NaCl solution.
toneally into rats weighing 200 to 220 g, 60 to 90 min before administration of glucagon or insulin. Actinomycin D, 0.66 μg per g of body weight, was injected intraperitoneally into rats weighing 200 to 250 g 2 hours before administration of glucagon or insulin. Control animals received injections of 0.9% NaCl solution but were otherwise treated identically with those pretreated with each drug.

Statistical analysis of the data was done using Student's t test (18). In all experiments, the means ± standard error for three to five rats are given.

RESULTS

Insulin injection into the portal vein caused a rapid, significant (p < 0.01) increase in rat hepatic phosphofructokinase (at 5, 10, and 20 min) and pyruvate kinase (at 5 and 10 min) activities, a significant (p < 0.01) decrease in fructose diphosphatase activity (at 5, 10, 20, and 40 min), but no change in fructose diphosphate aldolase activity or cyclic AMP concentrations (Fig. 1). The insulin effect was time-dependent, being detectable within 5 min, and was maximal 10 min following the injection of 0.06 unit (0.3 unit per kg). The enzyme activities then returned toward control values. Forty minutes after insulin injection, pyruvate kinase and phosphofructokinase activities were back to control levels, but fructose diphosphatase activity was still significantly (p < 0.01) different from control values. The enzyme activities and cyclic AMP (not shown) in control animals given an injection of the NaCl vehicle were not significantly altered during the 40-min test period (Fig. 1).

The insulin effect was dependent on the amount of insulin injected (Table I). No effect was detected with 0.005 unit per kg; 0.015 unit per kg and greater produced a significant (p < 0.01) change in phosphofructokinase (0.015, 0.15, and 1.5 units per kg), pyruvate kinase (0.15 and 1.5 units per kg), and fructose diphosphatase (0.15 and 1.5 units per kg) activities. Enzyme activities in the control animals given injections of the NaCl vehicle were unaltered. Puromycin and actinomycin D pretreatment did not alter the significant (p < 0.01) enzyme responses to 0.15 unit per kg of insulin (Table II). When glucagon (150 μg) was injected 5 min after the insulin injection (0.15 unit per kg), the significant (p < 0.01) insulin effect on the activities of phosphofructokinase, pyruvate kinase, and fructose diphosphatase was reversed over the next 5 to 10 min (Table III). This was accompanied by a significant (p < 0.01) increase in cyclic AMP concentration following the glucagon (Table III).

Glucagon produced a significant (p < 0.01) decrease in phosphofructokinase and pyruvate kinase activities and a significant (p < 0.01) increase in fructose diphosphatase activity within 2 to 10 min after injection into the portal vein (Fig. 2). These significant (p < 0.01) enzyme changes were maximal after 5 to 10 min and persisted for at least 20 min following a single injection of 300 μg. The glucagon effect on the enzymes was preceded by a significant (p < 0.01) increase in cyclic AMP concentration, which occurred within 30 s after injection of the hormone (Fig. 2). No effect of glucagon on fructose diphosphate aldolase was

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**Fig. 1.** The effect of 0.06 unit of insulin given via the portal vein on hepatic phosphofructokinase, fructose diphosphatase aldolase, pyruvate kinase, fructose diphosphatase, and cyclic AMP concentration.
TABLE I
Dose response of intravenous insulin on hepatic enzyme activities

Activities are expressed as nanomoles of substrate metabolized per min per mg of protein.

| Insulin dose (U/kg) | Time (min.) | Enzymes            |
|---------------------|-------------|--------------------|
|                     |             | Phosphofructokinase| Pyruvate kinase | Fructose-1,6-diphosphatase | Fructose-1,6-diphosphate aldolase |
| 0                   | 0           | 47 ± 4             | 171 ± 14       | 54 ± 4                      | 51 ± 4                          |
|                     | +4          | 46 ± 4             | 164 ± 13       | 54 ± 4                      | 50 ± 4                          |
|                     | +15         | 46 ± 3             | 163 ± 14       | 54 ± 4                      | 53 ± 4                          |
| 0.005               | 0           | 49 ± 4             | 174 ± 15       | 53 ± 4                      | 54 ± 3                          |
|                     | +4          | 47 ± 4             | 203 ± 13       | 54 ± 4                      | 54 ± 5                          |
|                     | +15         | 52 ± 3             | 159 ± 12       | 56 ± 4                      | 52 ± 5                          |
| 0.015               | 0           | 43 ± 4             | 184 ± 14       | 50 ± 3                      | 54 ± 4                          |
|                     | +4          | 57 ± 4             | 228 ± 12       | 55 ± 3                      | 52 ± 4                          |
|                     | +15         | 59 ± 4             | 236 ± 15       | 51 ± 3                      | 53 ± 4                          |
| 0.15                | 0           | 50 ± 4             | 163 ± 13       | 52 ± 3                      | 51 ± 4                          |
|                     | +4          | 70 ± 4             | 208 ± 13       | 37 ± 3                      | 52 ± 4                          |
|                     | +15         | 74 ± 5             | 238 ± 16       | 37 ± 3                      | 51 ± 4                          |
| 1.5                 | 0           | 49 ± 3             | 165 ± 14       | 58 ± 5                      | 56 ± 5                          |
|                     | +4          | 72 ± 5             | 232 ± 17       | 38 ± 4                      | 55 ± 3                          |
|                     | +15         | 79 ± 6             | 271 ± 17       | 35 ± 4                      | 54 ± 4                          |

a Mean ± S.E.M. for four rats per treatment group.
b Mean ± S.E.M. for five rats per treatment group.

*p < 0.05.

**p < 0.01.

detected. Injection of the glucagon vehicle had no effect on any of the enzyme activities (Fig. 2). The significant (p < 0.01) glucagon effect was dependent on the amount of hormone injected from about 15 μg to 300 μg (Table IV). No significant effects were seen with doses of 0.5 and 1.5 μg. The significant (p < 0.01) glucagon (150 μg) effect on these enzymes was unaltered by pretreatment of the rats with actinomycin D or puromycin (Table V). The significant (p < 0.01) glucagon (150 μg) effect on the enzyme activities was reversed when insulin (0.15 unit per kg) was injected 5 min after the glucagon (Table VI). This was accompanied by a significant (p < 0.01) decrease in cyclic AMP concentration 5 min following the insulin (Table VI).

Table VII shows the effects of intravenous insulin (0.15 unit per kg) on rat epididymal fat, renal cortex, and skeletal muscle glycogenolytic enzyme and fructose diphosphatase activities. Insulin produced a rapid and significant (p < 0.01) increase in phosphofructokinase and pyruvate kinase activities in each of the three tissues, whereas fructose diphosphatase aldolase activity was unchanged. In each of the three tissues, insulin produced a rapid and significant (p < 0.01) decrease in fructose diphosphatase activity.

The effect of intravenous glucagon (0.15 mg) on rat epididymal fat, renal cortex, and skeletal muscle glycogenolytic enzymes and fructose diphosphatase activities is shown in Table VIII. The responses of phosphofructokinase (p < 0.01) and fructose diphosphatase (p < 0.01) activities in epididymal fat were similar to those in the liver, whereas epididymal fat pyruvate kinase and fructose diphosphatase aldolase activities were unaffected by glucagon. In the renal cortex, glucagon significantly (p < 0.01) decreased pyruvate kinase activity and increased fructose diphosphatase activity, whereas fructose-1,6-diphosphatase activity was unchanged. The only enzyme significantly altered by glucagon in skeletal muscle was fructose diphosphatase, which was significantly increased (p < 0.01) at 4 min.
TABLE II

Effect of puromycin and actinomycin D on insulin (0.16 unit per kg)-induced changes in hepatic enzyme activities

Activities are expressed as nanomoles of substrate metabolized per min per mg of protein.

| Enzymes                  | Time (mins.) | Insulin | Insulin + puromycin | Insulin + actinomycin D |
|--------------------------|--------------|---------|---------------------|-------------------------|
|                          | 0            | 4       | 15                  | 0          | 4       | 15                  |
| Phosphofructokinase      | +4±3         | +3+4**  | +5+3**              | +5+4**      | +3+3**  |
| Pyruvate kinase          | 141±10       | 181±14* | 210±13**            | 139±12      | 184±15**| 209±11**            |
| Fructosediphosphatase    | 46±4         | 30±3**  | 27±3**              | 46±4        | 31±2**  | 27±3**              |
| Fructosediphosphate aldolase | 48±4     | 48±4   | 45±5                | 44±4        | 45±3    | 44±4                |
|                          |              |         |                     |             |         |                     |

*a Mean ± S.E.M. for three rats per treatment group.

* p < 0.05.

** p < 0.01.

TABLE III

Reversal by glucagon (150 µg) of insulin (0.16 unit per kg)-induced changes in hepatic enzyme activities and response of cyclic AMP

Enzyme activities are expressed as nanomoles of substrate metabolized per min per mg of protein. Cyclic AMP concentration is expressed as picomoles per mg of protein.

| Component                  | Minutes following treatment |
|----------------------------|-----------------------------|
|                            | 0a                          | 5b                       | 10                        | 15                        |
| Phosphofructokinase        | 47±3c                      | 62±4**                   | 50±2                      | 48±2                      |
| Pyruvate kinase            | 130±11c                    | 208±13**                 | 131±13                    | 126±12                    |
| Fructosediphosphatase      | 51±3c                      | 37±3**                   | 48±3                      | 51±3                      |
| Fructosediphosphate aldolase | 56±5c                 | 57±5                     | 56±5                      | 57±5                      |
| Cyclic AMP                 | 21±5d                      | 15±5                     | 192±51**                  | 102±75**                  |

*a Insulin (0.15 U/kg) injected immediately after zero time samples obtained.

*b Glucagon (150 µg) injected immediately after taking 5 minute samples.

c Mean ± S.E.M. for 4 rats per treatment group.

d Mean ± S.E.M for 3 rats per treatment group.

** p < 0.01.

Cyclic AMP (0.05 mmole per kg) given intravenously produced the same significant (p < 0.01) effect as glucagon on the hepatic enzyme activities (Table IX). Phosphofructokinase and pyruvate kinase activities decreased significantly (p < 0.01) within 5 min, were lowest at 10 min, and returned to normal by 15 min. Fructose diphosphatase activity increased significantly (p < 0.01) within 5 min and remained elevated for at least 15 min. No change was seen in fructose diphosphate aldolase activity.

The in vivo effects of intravenous cyclic AMP on epididymal fat, renal cortex, and skeletal muscle enzymes are shown in Table IX. Cyclic AMP injection produced changes identical with those produced by glucagon in enzyme activities in the renal cortex and skeletal muscle. In the renal cortex, cyclic AMP significantly (p < 0.01) increased fructose diphosphatase activity, significantly (p < 0.01) decreased pyruvate kinase activity, but had no significant effect upon the other two enzyme activities. In skeletal muscle, the only enzyme significantly altered by cyclic AMP was fructose diphosphatase, which increased about 2-fold at 10 min postinjection. Cyclic AMP injections significantly (p < 0.01) decreased epididymal fat pyruvate kinase and phosphofructokinase activities, increased fructose diphosphatase activity, and had no significant effect upon the fructose diphosphate aldolase activity.
When a breakdown product of cyclic AMP, 5'-AMP (0.05 mmole per kg), was injected intravenously, there was no significant change in the activities of phosphofructokinase, pyruvate kinase, or fructose diphosphate aldolase (Table X). Although the mean value of pyruvate kinase was decreased at 5 min and increased at 15 min, these changes were not statistically significant. However, fructose diphosphatase activity was significantly (p < 0.05) decreased after 5 min but was the same as the control after 10 and 15 min (Table X).

Glucagon (1.0 mg per ml) and insulin (1.0 mg per ml) added to the 104,000 × g supernatant had no effect on the activities of any of the enzymes. In all animals, glucagon (15 to 300 µg) produced a rise in plasma glucose levels and insulin (0.005 to 1.5 units per kg) caused a drop in plasma glucose levels.

**DISCUSSION**

The antagonistic effects of insulin and glucagon on glycogen metabolism and glycolysis and gluconeogenesis in liver are well known, but the mechanisms by which these hormones produce their effects are not completely understood. In recent years these hormones have been found to exert their opposing effects on glycogen metabolism by controlling the activity of phosphorylase and glucogen synthetase through phosphorylation-dephosphorylation mechanisms. It seems clear that the glucagon effect is mediated by cyclic AMP and that this nucleotide regulates the activity of glycogen synthetase and phosphorylase by activating a protein kinase (1). A series of phosphorylations then occurs, finally resulting in phosphorylation of these two regulatory enzymes. Phosphorylase activity increases and glycogen synthetase activity decreases with phosphorylation.

Insulin, on the other hand, increases glycogen synthetase activity and decreases phosphorylase activity, although the mechanism is less well understood. Insulin rapidly increases the activity of a phosphatase which dephosphorylates glycogen synthetase (19). This effect does not seem to be mediated by a decrease in the cyclic AMP level. Although insulin does decrease cyclic AMP levels in the liver under certain conditions, it does not usually cause a drop below basal levels. It has, therefore, been suggested that insulin action might be mediated by a chemical messenger other than cyclic AMP and that this mediator could affect the cyclic AMP system (20, 21).

The mechanisms by which insulin increases glycolysis and decreases gluconeogenesis (22–26) and by which glucagon produces the opposite effects on these pathways (24, 25) have not
Table IV

Dose response of intravenous glucagon on hepatic enzyme activities

Activities are expressed as nanomoles of substrate metabolized per min per mg of protein.

| Glucagon dose (µg) | Time (min.) | Phosphofructokinase | Pyruvate kinase | Fructose-diphosphatase | Fructose-diphosphate aldolase |
|-------------------|-------------|---------------------|----------------|------------------------|-----------------------------|
| 0 a               | 0           | 43 ± 3 b            | 163 ± 13       | 53 ± 4                 | 53 ± 4                      |
|                   | 4           | 41 ± 3              | 165 ± 12       | 53 ± 4                 | 55 ± 3                      |
|                   | 15          | 42 ± 3              | 166 ± 13       | 54 ± 4                 | 55 ± 3                      |
| 0.5               | 0           | 41 ± 3              | 171 ± 15       | 52 ± 4                 | 52 ± 4                      |
|                   | 4           | 41 ± 3              | 140 ± 12       | 50 ± 5                 | 54 ± 4                      |
|                   | 15          | 37 ± 3              | 151 ± 13       | 69 ± 4                 | 53 ± 4                      |
| 1.5               | 0           | 41 ± 3              | 155 ± 14       | 53 ± 4                 | 53 ± 4                      |
|                   | 4           | 40 ± 3              | 138 ± 13       | 58 ± 5                 | 55 ± 5                      |
|                   | 15          | 40 ± 2              | 154 ± 14       | 60 ± 4                 | 54 ± 4                      |
| 15                | 0           | 42 ± 3              | 141 ± 13       | 51 ± 4                 | 51 ± 3                      |
|                   | 4           | 28 ± 2**            | 108 ± 11       | 59 ± 4                 | 52 ± 4                      |
|                   | 15          | 26 ± 2**            | 81 ± 7**       | 63 ± 4*                | 52 ± 4                      |
| 150               | 0           | 42 ± 3              | 158 ± 14       | 52 ± 4                 | 54 ± 5                      |
|                   | 4           | 20 ± 2**            | 42 ± 4**       | 66 ± 4*                | 55 ± 4                      |
|                   | 15          | 29 ± 1**            | 47 ± 5**       | 69 ± 6**               | 53 ± 4                      |
| 300               | 0           | 43 ± 4              | 143 ± 13       | 54 ± 4                 | 58 ± 5                      |
|                   | 4           | 22 ± 2**            | 53 ± 5**       | 71 ± 6**               | 58 ± 5                      |
|                   | 15          | 30 ± 1**            | 48 ± 5**       | 77 ± 6**               | 55 ± 4                      |

a Injection of glucagon vehicle only (1.6% glycerin and 0.2% phenol).
b Mean ± S.E.M. for 4 rats per treatment group.

* p < 0.05.
** p < 0.01.
TABLE V

Effect of puromycin and actinomycin D on glucagon (160 µg)-induced changes in hepatic enzyme activities

Activities are expressed as nanomoles of substrate metabolized per min per mg of protein.

| Time (mins.) | Glucagon | Glucagon + puromycin | Glucagon + actinomycin D |
|--------------|----------|----------------------|-------------------------|
|              |          |                      |                         |
| Enzymes      |          |                      |                         |
| Phosphofructokinase | 42±3     | 27±3**               | 26±2**                  |
| Pyruvate kinase        | 160±15   | 68±8**               | 58±8**                  |
| Fructosediphosphatase  | 42±2     | 51±2                 | 57±3**                  |
| Fructosediphosphate   | 47±4     | 44±3                 | 45±3                    |

a Mean ± S.E.M. for three rats per treatment group.

* p < 0.05.

** p < 0.01.

TABLE VI

Reversal by insulin (0.16 unit per kg) of glucagon (160 µg)-induced changes in hepatic enzyme activities and response of cyclic AMP

Enzyme activities are expressed as nanomoles of substrate metabolized per min per mg of protein. Cyclic AMP concentration is expressed as picomoles per mg of protein.

| Component                  | 0a        | 5b        | 10         | 15         |
|----------------------------|-----------|-----------|------------|------------|
| Phosphofructokinase        | 50±2c     | 36±1**    | 50±2       | 51±1       |
| Pyruvate kinase            | 151±14    | 71±8**    | 118±11     | 118±10     |
| Fructosediphosphatase      | 48±3      | 63±4**    | 53±2       | 45±3       |
| Fructosediphosphate aldolase | 53±4     | 52±4      | 52±4       | 53±4       |
| Cyclic AMP                 | 12±3      | 92±5**    | 51±9**     | 29±4**     |

a Glucagon (150 µg) injected immediately after zero time samples obtained.

b Insulin (0.15 U/kg) injected immediately after taking 5 minute samples.

c Mean ± S.E.M. for 3 rats per treatment group.

* p < 0.05.

** p < 0.01.

in the control of gluconeogenesis by this hormone. Other investigators have recently presented indirect evidence that glucagon can increase fructose diphosphatase activity (32, 33). It is possible, therefore, that under certain conditions fructose diphosphatase may be rate-limiting in the gluconeogenic pathway. This is supported by the finding that low activity of this enzyme in human liver is associated with hypoglycemia (34-37).

Preliminary findings from our laboratory suggest that two other key enzymes in the gluconeogenic pathway, pyruvate carboxylase and phosphoenolpyruvate carboxykinase, are not rapidly altered by glucagon and insulin in vivo.1

The glucagon effect in the liver on phosphofructokinase, pyruvate kinase, and fructose diphosphatase was preceded by a significant increase in cyclic AMP concentration and suggests that the effect is mediated by this nucleotide. Additional support for this suggestion includes the fact that cyclic AMP mimics the effect of glucagon on gluconeogenesis in the isolated perfused rat liver (25, 31). Epinephrine, the action of which is mediated by cyclic AMP, given in vivo, causes changes in the activities of these enzymes similar to those produced by glucagon (28). Finally, cyclic AMP injected into the rat portal vein produced changes identical with those seen with glucagon, whereas 5'-AMP had only a minimal, and opposite, effect on fructose diphosphatase.

The lack of correlation between the effect of insulin on these enzymes and a change in cyclic AMP suggests that the insulin
TABLE VII

Effect of intravenous insulin (0.16 unit per kg) on epididymal fat, renal cortical, and skeletal muscle enzyme activities

Activities expressed as nanomoles of substrate metabolized per min per mg of protein.

| Time (mins.) | Epididymal fat | Renal cortex | Skeletal muscle |
|-------------|----------------|--------------|-----------------|
| Enzymes     |                |              |                 |
| Phosphofructokinase | 2.3±0.2** | 3.3±0.2** | 14.5±1.1**      |
| Pyruvate kinase            | 8.8±0.7   | 14.5±0.6**  | 34.8±2.9        |
| Fructosediphosphatase      | 1.6±0.2   | 0.9±0.1**   | 26.0±2.4        |
| Fructosediphosphate aldolase | 2.4±0.3  | 2.5±0.4     | 28.9±2.4        |

a Mean ± S.E.M. for 4 rats per treatment group.

* p < 0.05.

** p < 0.01.

TABLE VIII

Effect of intravenous glucagon (160 μg) on epididymal fat, renal cortical, and skeletal muscle enzyme activities

Activities are expressed as nanomoles of substrate metabolized per min per mg of protein.

| Time (mins.) | Epididymal fat | Renal cortex | Skeletal muscle |
|-------------|----------------|--------------|-----------------|
| Enzymes     |                |              |                 |
| Phosphofructokinase | 2.6±0.2** | 1.5±0.1**  | 17.8±1.8        |
| Pyruvate kinase            | 9.8±1.8   | 10.2±0.3**  | 40.8±3.7        |
| Fructosediphosphatase      | 1.6±0.1   | 2.9±0.3**   | 22.6±2.3        |
| Fructosediphosphate aldolase | 2.6±0.3  | 2.7±0.3     | 30.9±2.8        |

a Mean ± S.E.M. for 4 rats per treatment group.

* p < 0.05.

** p < 0.01.

effect is not mediated by this nucleotide. However, the possibility has not been excluded that the free (unbound) concentration of intracellular cyclic AMP was altered by insulin or that there was a change in cyclic AMP concentration in a specific intracellular compartment (38). The reversal of the glucagon effect when insulin was injected 5 min after glucagon was accompanied by a decrease in the elevated cyclic AMP level produced by glucagon. This lowering effect of insulin on elevated cyclic AMP levels produced by glucagon is similar to that reported by others (28, 39).

The mechanism by which these hormones rapidly alter the activity of these enzymes is not known. However, it seems sufficiently plausible to consider tentatively that mechanisms similar to those involved in the control of glycogen synthetase and phosphorylase, i.e. phosphorylation-dephosphorylation, might exist. This would indicate that, through the same mechanisms, insulin and glucagon reciprocally regulate carbohydrate metabolism in several opposing pathways. Although this possibility is most attractive, other possibilities should be considered; these include (a) allosteric effects of cyclic AMP on the enzymes and (b) alteration of the concentration of certain metabolic intermediates, such as fatty acids, which alter the activity of these enzymes in vitro (43). The latter possibility seems unlikely, since the intermediates would be diluted 10- to 200-fold in concentration in the 104,000 x g supernatant during the analysis of the enzyme activities. No single metabolic inter-
**TABLE IX**

**Effect of intravenous cyclic AMP on hepatic, epididymal fat, renal cortical, and skeletal muscle glycolytic and fructose diphosphatase enzyme activities**

Activities are expressed as nanomoles of substrate metabolized per min per mg of protein.

| Tissue     | Enzymes Assayed           | Minutes Following Injection | 0   | +5   | +10  | +15  |
|------------|---------------------------|----------------------------|-----|------|------|------|
| Liver      | Phosphofructokinase       |                            | 45.1| 26.6 | 20.5 | 40.3 |
|            | Pyruvate kinase           |                            | 149.9| 100.0| 89.1 | 144.0|
|            | Fructosediphosphatase     |                            | 46.7| 98.7 | 96.7 | 100.1|
|            | Fructosediphosphate aldolase |                        | 51.0| 50.6 | 50.5 | 50.5 |
| Fat        | Phosphofructokinase       |                            | 2.6 | 1.6  | 1.2  | 2.0  |
|            | Pyruvate kinase           |                            | 8.6 | 5.6  | 5.7  | 8.4  |
|            | Fructosediphosphatase     |                            | 1.3 | 3.1  | 3.6  | 4.1  |
|            | Fructosediphosphate aldolase |                        | 2.9 | 2.9  | 2.8  | 3.0  |
| Renal cortex| Phosphofructokinase       |                            | 16.1| 17.2 | 16.9 | 17.8 |
|            | Pyruvate kinase           |                            | 30.4| 27.4 | 16.6 | 16.2 |
|            | Fructosediphosphatase     |                            | 25.8| 71.2 | 51.8 | 41.8 |
|            | Fructosediphosphate aldolase |                        | 30.4| 29.7 | 28.9 | 29.6 |
| Muscle     | Phosphofructokinase       |                            | 175.1| 164.2| 153.1| 146.2|
|            | Pyruvate kinase           |                            | 199.1| 2126.1| 2460.0| 2467.0|
|            | Fructosediphosphatase     |                            | 3.6 | 6.0  | 7.1  | 5.3  |
|            | Fructosediphosphate aldolase |                        | 53.4| 53.4 | 53.4 | 53.4 |

* Mean ± S.E.M. for three rats per treatment group. Activities expressed as nmols substrate metabolized/minute/mg protein.

* * p < 0.05.

**TABLE X**

**Effect of 6'-AMP (0.05 mmole per kg of body weight) given via the portal vein on hepatic enzyme activities**

Activities are expressed as nanomoles of substrate metabolized per min per mg of protein.

| Enzymes          | Time (mins.) |
|------------------|--------------|
|                  | 0            | 5            | 10           | 15           |
| Phosphofructokinase | 49 ± 4*     | 48 ± 3       | 45 ± 4       | 48 ± 3       |
| Pyruvate kinase   | 168 ± 16     | 146 ± 12     | 175 ± 15     | 230 ± 25     |
| Fructosediphosphatase | 50 ± 2     | 40 ± 2*      | 46 ± 3       | 48 ± 3       |
| Fructosediphosphate aldolase | 53 ± 4    | 54 ± 4       | 53 ± 4       | 55 ± 5       |

* Mean ± S.E.M. for 3 rats per treatment group.

* * p < 0.05.

mediate is known at present which would account for all of the enzyme changes seen after the hormone injections. Particularly, it is difficult to envision one or more ligands generated by a hormone the action of which can be reversed by the antagonistic hormone.

In this connection, the work of Mansour (44), concerning the effect of epinephrine on rabbit skeletal muscle phosphofructokinase, should be considered. Mansour (44) found it essential to prepare his muscle extracts in the presence of caffeine in order to demonstrate the difference between the control and epinephrine-treated muscle enzyme. A concentration of 4.25 × 10^-4 M cyclic AMP was also included in his assay mixture. The stimulatory effect of cyclic AMP and glucose 1,6-diphosphate on rabbit skeletal muscle phosphofructokinase required the presence of caffeine. It may well be that his epinephrine effect was related to the generation by epinephrine of one or more substances (ligands) which overcame the inhibitory effect of caffeine.

In our studies, intravenous glucagon and cyclic AMP had no...
effect on rat skeletal muscle phosphofructokinase at a time when significant changes were occurring in hepatic phosphofructokinase, whereas insulin did stimulate phosphofructokinase in muscle and liver. At least with regard to glucagon and cyclic AMP, no effect on skeletal muscle phosphofructokinase was found which would be consistent with the findings of Mansour (44) in the experiments in which caffeine was not used. Although we saw a small, significant change in skeletal muscle fructose diphosphatase activity following intravenous cyclic AMP, this may be of no physiological significance. The studies with cyclic AMP suggest that muscle enzymes are not particularly responsive to cyclic AMP, because cyclic AMP is not generated by glucagon, or it does not enter muscle readily, or it is rapidly destroyed despite the injection of cyclic AMP.

One may conclude from the studies of Mansour (44) that ligands generated by hormones may stimulate or inhibit enzyme activity. Conceivably, then, physiologically generated substances (ligands) might be implicated in the hormone-induced enzyme changes. In many in vitro studies in which ligands have been shown to have direct effects on enzymes, the concentrations used have been higher than those usually found physiologically intracellularly (e.g. in liver, cyclic AMP levels range from 10 to 20 pmoles per mg of protein).

Fructose diphosphatase and phosphofructokinase activities have been reported to be altered by cyclic AMP in vitro. Fructose diphosphatase activity from swine kidney was decreased by 0.6 mM cyclic AMP (45), and phosphofructokinase activity from liver fluke and sheep heart was increased by cyclic AMP (44, 46). These effects are opposite to those we observed with glucagon and cyclic AMP in vivo. The reason for these differences is not clear but may be related to the type of tissue, different species, and different conditions used in the different studies. When we add 1 mM cyclic AMP to the 104,000 × g supernatant from rat liver we observe a decrease in fructose diphosphatase activity.

Insulin and glucagon affect certain enzymes of extrahepatic tissues (epididymal fat, renal cortex, and skeletal muscle) very much like that in the liver. In each of the four tissues, intravenous insulin produced similar effects. Insulin significantly increased the activities of phosphofructokinase and pyruvate kinase, significantly decreased the activity of fructose diphosphatase, and had no effect upon fructose diphosphatase aldolase activity. The responses of the four enzymes to glucagon were not as consistent in each of the four tissues as those seen with insulin. This is not surprising, however, since glucagon is not known to have physiological effects in each of these tissues. Two of the four enzymes (fructose diphosphatase aldolase and fructose diphosphatase) did respond uniformly in each of the four tissues. The fructose diphosphatase aldolase activity was unchanged in each of the four tissues following glucagon injection.

In the extrahepatic tissues, glucagon produced a rapid and significant rise in fructose diphosphatase activity, which was mimicked by cyclic AMP. It is interesting that, even in the two tissues which are non-glucoseogenic (fat and skeletal muscle), glucagon still exerted a significant effect upon fructose diphosphatase activity. However, the activity of the enzyme in these two tissues is so low that the significance of the glucagon effect on fructose diphosphatase activity is not apparent. Glucagon produced a significant decrease in pyruvate kinase activity in renal cortex but had no effect upon fat or skeletal muscle pyruvate kinase activity. In fat, glucagon significantly decreased the activity of phosphofructokinase. Phosphofructokinase activity was unchanged in the renal cortex and skeletal muscle.

Insulin produced uniform changes in the enzymes studied in all of the tissues tested, whereas glucagon did not. The lack of responsiveness of certain tissues to glucagon might be a reflection of the isozyme distribution of pyruvate kinase and phosphofructokinase. Based upon the immunoreactivity of the two isozyme forms, the L (liver), or adaptive, form of pyruvate kinase has been found only in rat liver and erythrocytes (47). However, despite the failure of neutralization with the type L antibody, the crude kidney pyruvate kinase showed two peaks on starch zone electrophoresis, one of which had the same mobility as the type L enzyme (47). Since pyruvate kinase in the two glucagon-responsive tissues (liver and kidney) probably contains both the L and M isozyme forms whereas the pyruvate kinase in the two non-glucagon-responsive tissues exists only in the M form (47), it would seem that glucagon exerts its effect upon the L form of the enzyme. Presumably, liver and adipose tissue may possess inducible forms of phosphofructokinase (called the L, or liver, form), whereas the skeletal muscle and kidney do not.

In recent years it has been shown that the activity of key enzymes in other metabolic pathways is altered by phosphorylation. These include the hormone-sensitive lipase in adipose tissue (48), acetyl-CoA carboxylase (49), and pyruvate dehydrogenase (50). Certainly these results provide sufficient precedent to make consideration of a phosphorylation-dephosphorylation mechanism most tempting. Nevertheless, at least in the case of skeletal muscle phosphofructokinase, attempts to demonstrate such a mechanism have not been successful (44).

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