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D. THE STIMULATION OF GLUCONEOGENESIS FROM LACTATE BY EPINEPHRINE, GLUCAGON, AND CYCLIC 3',5'-ADENYLATE IN THE PERFUSED RAT LIVER[1]

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The stimulation of gluconeogenesis by glucagon was first suggested by the observation of Kalant (7) that administration of the hormone to the intact animal increased urinary nitrogen excretion. Rose and Nelson (12) showed that crude glucagon preparations elevated the blood urea in nephrectomized rats. Salter *et al.* (13) found increased urinary nitrogen and urea excretion following chronic glucagon administration to the rat and were the first to suggest that the hormone affected gluconeogenesis. Izzo and Glasser (3, 5, 6) confirmed and extended these findings. Shoemaker and Van Itallie (15) demonstrated increased amino acid uptake by the liver in dogs infused with glucagon. More recently, Miller (8, 9) found that physiological concentrations (18) of glucagon stimulated protein catabolism and urea production in the isolated perfused rat liver. Schimassek and Mitzkat (14) showed that physiologic levels of glucagon stimulated lactate utilization by the liver and obtained presumptive evidence that conversion to glucose had been increased. They observed, furthermore, that the hormone reduced the level of fructose diphosphate and increased hexose monophosphate in the tissue. They concluded that this part of the Embden-Meyerhof pathway was under the control of glucagon.

Epinephrine (E) was also tested in a number of the above studies with conflicting results. Rose and Nelson (12) reported a rapid rise in blood urea following intravenous infusion of the catecholamine in rats. Izzo and Glasser (5) on the other hand, did not find any rise in nitrogen excretion in rats receiving repeated injections of E in oil over several days. Also Miller (9) could not observe increased urea production by the perfused liver exposed to very high levels of E although glycogenolysis was strongly stimulated. Schimassek and Mitzkat (14) concluded, however, that E promoted lactate and pyruvate utilization by the isolated liver but did not find significant changes in the tissue levels of hexose phosphates like those seen with glucagon.

In the present study, we have attempted first to establish the effects of glucagon and catecholamines on gluconeogenesis in a more direct and quantitative manner. For this purpose, we have measured the conversion of C^{14}-lactate to labeled glucose and glycogen in the isolated, perfused rat liver. Second, we have tried to define the points of action of these hormones on gluconeogenesis. Our conclusions in this regard are based on measurements of the concentrations of intermediates in the metabolic pathway from lactate to glucose.

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METHODS

Livers of male Sprague-Dawley rats weighing 90 to 140 g were perfused by the method of Mortimore (10). The perfusion medium consisted of Krebs bicarbonate buffer containing 3% (w/v) of bovine serum albumin and about 20% (v/v) of washed rat erythrocytes. L-Lactate and C^{14}-DL-lactate were the only substrates added. The medium was recirculated through the liver at a rate of about 1.5 ml per g wet weight of tissue per minute at 37°C.

At the end of the perfusion, the liver tissue was fixed by freeze-clamping (21), extracted with perchloric acid at −10°, and neutralized with K_2CO_3. Lactate and all intermediates were assayed enzymatically (1). Glucose was determined routinely by the ferricyanide procedure as modified for use with the Technicon AutoAnalyzer (see manual of the Technicon Instruments Corp., Chauncey, N.Y.) Results were spot checked by the glucose oxidase procedure (see 1). C^{14}-Glucose was counted after removal of lactate and other radioactive ions by exhaustive shaking of the deproteinized perfusate with a mixture of Dowex 50, H† form and Duolite A-4, OH^- form. Details of the procedures, methods and calculations will be published later.

Crystalline glucagon was kindly supplied by Eli Lilly Co., Indianapolis, Indiana. *l*-Epinephrine bitartrate was a commercial preparation (K and K Laboratories, Inc., Jamaica 33, N. Y.).

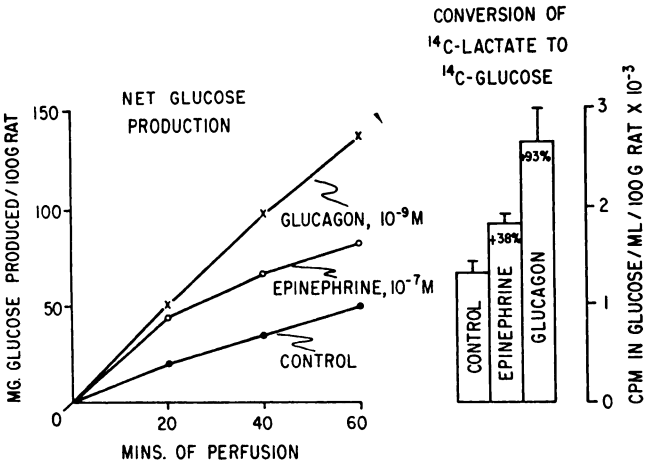

FIG. 1. Effects of glucagon and epinephrine on glucose production and gluconeogenesis from C^{14}-lactate in the perfused liver from fed rats. The panel on the left shows net glucose production and, on the right, the relative amounts of labeled glucose in the perfusate after 1 hr.

The medium contained initially 20 mM L-lactate and C^{14}-DL lactate (10,350 cpm/ml). The concentration of D-lactate was less than 0.001 mM, so low that presumably little radioactivity from this isomer was converted to glucose. Estimates in the text are based on this assumption, but the matter is not critical to any of the general conclusions. Glucagon or E were added to the medium to give initially the concentrations shown. An equal quantity of hormone was infused at constant rate into the medium during the hour.

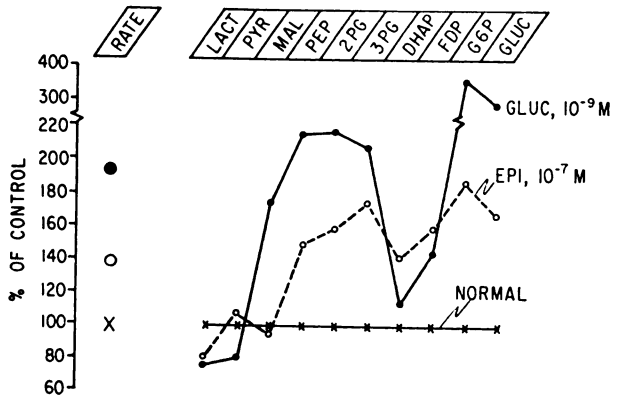

FIG. 2. Effect of glucagon and E on the rate of gluconeogenesis and levels of intermediates in the pathway of lactate to glucose.

The experimental conditions are described in figure 1 and in the text. Absolute values for the controls, expressed as mμmoles/g dry weight of liver, were as follows: lactate, 21,500; pyruvate, 1520; malate, 1,670; phosphopyruvate, 420; 2-phosphoglycerate, 120; 3-phosphoglycerate, 910; dihydroxyacetone phosphate, 68; α-glycerophosphate, 930; fructose-diphosphate, 33; glucose-6-phosphate, 302.

Rates of utilization and production of substances are expressed per 100 g of body weight of the liver donor. The values in the figures are the means of 6 or more experiments.

RESULTS

The effects of glucagon and E on glucose production were examined in livers from rats fed *ad lib.* The medium contained 20 mM L-lactate, which is approximately 10 times the concentration for half maximum gluconeogenesis in this system. In the control series (left panel), glucose was produced at a nearly linear rate over 1 hr (fig. 1). The fraction of this glucose which was formed from lactate could be estimated from the incorporation of C^{14} of lactate into the perfusate glucose (right panel). Up to 40% of the glucose came from this source, the remainder arising presumably from glycogenolysis. Addition of glucagon caused a marked stimulation of glucose production and doubled the conversion of lactate to glucose. Addition of E caused nearly as great an increase in glucose production over the first 20 min but the rate fell off thereafter. We have not yet tested higher concentrations to know whether the fall off was due to a loss of sensitivity or to destruction of the hormone at a rate faster than it was being infused into the medium. E caused a substantial increase in the conversion of C^{14}-lactate to glucose. An effect was not obtained with 10^{-8} M E but the true threshold concentration is difficult to assess since the rate of hormone destruction in this system is not known. In a few experiments, norepinephrine (NE) at 10^{-7} M appeared to be as effective as the same concentration of E.

In all of these experiments there was a net loss of glycogen by the liver and the C^{14} incorporated into glycogen was negligible compared to that found in glucose.

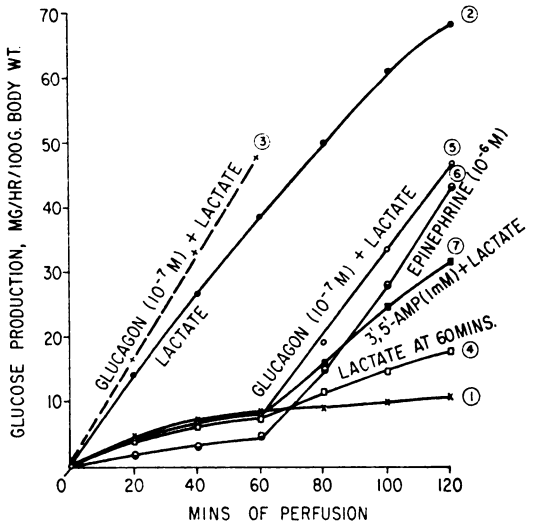

FIG. 3. Effect of glucagon, E and cyclic 3′,5′-AMP on gluconeogenesis from lactate in the perfused liver from fasted rats. In experiments shown by curves 2 and 3, 20 mM C^{14}-lactate was present in the perfusion medium from the start. In all other experiments lactate was not added before 60 min.

In order to define the points of hormone action in the metabolic pathway from lactate to glucose the levels of the intermediates were estimated at the end of the perfusion. For simplicity of presentation, the values from the livers of the control rats are plotted as 100% (fig. 2). With glucagon added to the medium, the rate of gluconeogenesis from C^{14}-lactate was about doubled as seen on the left of the figure. The levels of lactate and pyruvate in the tissue were reduced as would be expected with greater utilization. On the other hand, malate and phosphopyruvate (MAL and PEP in the figure) were considerably elevated. Malate probably lies outside the pathway of lactate to glucose (see 20) but is thought to be in equilibrium with the true intermediate, oxaloacetate. The latter compound could not be measured easily in liver and we have assumed that changes in malate would reflect changes in oxaloacetate.[2] On this basis, it seemed probable that the conversion of pyruvate to oxaloacetate had been stimulated since the enzyme responsible, pyruvate carboxylase,[3] was able to maintain a faster turnover of substrate despite a lower than normal substrate concentration

[2] This assumption rests on the following arguments. First, the malate oxaloacetate equilibrium is catalyzed by malate dehydrogenase. According to the estimates of Weber *et al.* (20) in liver homogenates, this enzyme has a greater capacity than any of those in the pathway of lactate to glucose. Thus interconversion of the compounds is not likely to be limited by insufficient enzyme. Second, malate dehydrogenase requires NAD. Thus any sustained departure from the malate:oxaloacetate equilibrium might be reflected by a change in the NADH:NAD^+ of the cytoplasm. The latter can be estimated by the α-glycerophosphate:dihydroxyacetone phosphate couple according to Hohorst, Kreutz and Bücher (17). In the experiments of fig. 2, the ratios of α-glycerophosphate:dihydroxyacetone were as follows: control, fed, 13.7; glucagon, 12.5; and E 11.5. The approximate constancy of these ratios supports the assumption of a constant malate:oxaloacetate ratio.

[3] Pyruvate:CO_2 ligase [ADP], EC 6.4.1.1.

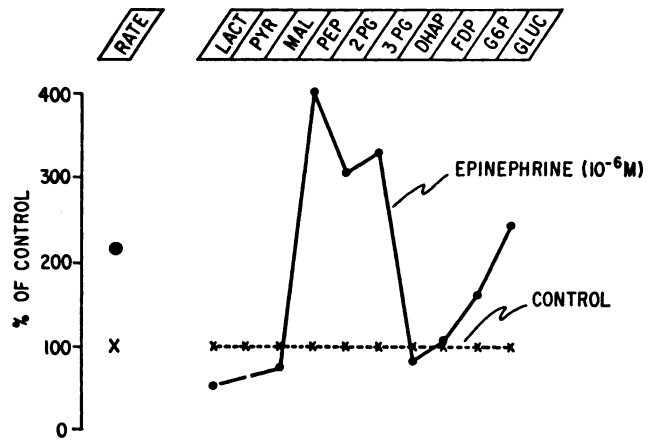

FIG. 4. Effect of E on the rate of gluconeogenesis and on the tissue levels of intermediates in the pathway from lactate to glucose. The experiments are taken from those shown in figure 3 (curves 4 and 6). The rate of gluconeogenesis was measured by the production of labeled glucose from C^{14}-lactate in the medium. Control values from livers perfused with lactate only are plotted as 100%.

and a higher than normal concentration of product. Similarly, the conversion of oxaloacetate to phosphopyruvate was stimulated; and this suggests activation of phosphopyruvate carboxylase.[4] The sharp drop in concentration of metabolites between 3-phosphoglycerate (3 PG) and dihydroxyacetone phosphate (DHAP) would suggest an inhibition of this step were it not that the flow of substrate was almost twice the control rate. The low level of dihydroxyacetone phosphate is probably explained by a faster rate of utilization. Activation of a step in its utilization is suggested by the high concentration of glucose-6-phosphate relative to fructose diphosphate. This change, which is the same as that noted by Schimassek and Mitzkat (14), could be most easily explained by a stimulation of fructose diphosphatase.

With addition of E to the medium, the pattern was similar to that with glucagon but the changes were less marked, as might be expected from the lower rate of gluconeogenesis. The largest, and perhaps only significant change occurred between malate and phosphopyruvate; this would suggest increased phosphopyruvate carboxylase activity.

In livers from rats fasted 20 hr, glycogen was markedly depleted and, as a consequence, the rate of glucose production was very slow when perfusions were carried out without lactate or other glucogenic substrates in the medium (curve 1, fig. 3). With 20 mM lactate, however, glucose production was substantial (curve 2), gluconeogenesis being about twice that seen in the livers from fed rats. Glucagon stimulated the rate appreciably (curve 3). When the livers were first perfused without substrate for 1 hr, and lactate was then added, the rate of gluconeogenesis remained very low (curve 4). Under these circumstances, glucagon elevated gluconeogenesis to the maximum rate (curve 5). Concentrations

[4] GTP:Oxaloacetate carboxy-lyase [transphosphorylating], EC 4.1.1.32.

of hormone in the range of 1×10^{-10} M were effective. E (1×10^{-6} M) had the same effect as glucagon (curve 6). Lower concentrations of catecholamine were not tested. Cyclic 3′,5′-AMP also stimulated gluconeogenesis but a concentration of 1×10^{-3} M was necessary (curve 7) to obtain this effect.

In these experiments with liver of fasted rats, the incorporation of labeled carbon into glycogen was negligible.

Analyses of intermediates were carried out as described for figure 2. The patterns produced by glucagon, E and cyclic 3′,5′-AMP were qualitatively the same. The results with E are shown in figure 4. The most striking feature is the marked rise in substrate level between malate and phosphopyruvate; this suggests, as before, an activation of phosphopyruvate carboxylase. The fall between 3-phosphoglycerate and dihydroxyacetone phosphate also suggests activation of steps utilizing the latter compound as noted earlier in connection with figure 2. The conversion of glucose-6-phosphate to glucose, however, would seem to be as much affected as that of fructose diphosphate to hexose monophosphate.

DISCUSSION

A stimulation of gluconeogenesis by glucagon and E has been shown unequivocally in the present study by the increased production of labeled glucose from C^{14}-lactate. The question arises whether this effect is physiologically important.

As regards glucagon, the lowest fully effective concentration tested (0.7 μg/liter) was within the range of hormone concentrations found by Unger and Eisentraut (18) in human plasma. In our experience, the sensitivity of the gluconeogenic and glycogenolytic processes appears to be about the same. Equal sensitivity would seem desirable teleologically in most situations requiring increased glucose production since the two processes would reinforce each other. Elevated glucagon concentrations in the plasma have been shown by Unger and Eisentraut (18) in hypoglycemia and in fasting. In the latter condition, the stimulation of gluconeogenesis by glucagon could have particular importance since glycogen reserves are low and could not provide a continuing supply of glucose.

With regard to E, we did not attempt to determine the minimum effective concentration for gluconeogenesis. E, or NE, however, in a concentration of 10^{-7} M gave a strong response, and this level (18 μg/liter) is not greatly above the level (6 μg/liter) found in rats exposed to stress (see 19). It is probable, however, that blood levels of the hormone are probably less important than the release of catecholamine at nerve endings in the liver. In this connection it is known that stimulation of the hepatic nerve elevates the blood glucose. In view of our results, the hyperglycemia could be due to a gluconeogenic as well as to a glycogenolytic response.

The importance of lactate as a precursor of glycogen and blood glucose has not been fully evaluated. The resting concentration of lactate in the blood is about 1 mM, which is about one half of the K_m for lactate gluconeogenesis according to our studies (unpublished). On this basis, lactate could give rise to

substantial quantities of glucose and glycogen. A very rapid incorporation of C^{14}-lactate into blood glucose and tissue glycogen of the rat is also suggested by recent, unpublished observations in this laboratory (L. S. Jefferson, 1964). In this connection, an important role for catecholamines may be proposed as a part of the Cori cycle. With muscular exercise, or in situations of acute stress which cause the release of large quantities of lactate into the blood, the amines may accelerate the reconversion of lactate to blood glucose. This would be important for maintaining substrate for continued energy production by rapid glycolysis in the muscles.

The carbon of the gluconeogenic amino acids enters the metabolic pathway from lactate to glucose through pyruvate or oxaloacetate. Stimulation of the pathway could therefore increase utilization of amino acids and would account for the observations on increased nitrogen excretion cited in the introduction. A possibility remains, of course, that gluconeogenesis from amino acids may be limited by steps prior to the pathway investigated in this study.

The similarity in the patterns to intermediates suggest that the catecholamines and glucagon have common sites of action. Since the same gluconeogenic effect and pattern of intermediates are also produced by cyclic 3′,5′-AMP (J. H. Exton, to be published) it seems most likely that this substance is the intracellular mediator for both hormones (17). Assays for cyclic 3′,5′-AMP in the liver were very kindly carried out for us by Dr. R. W. Butcher of this laboratory and showed approximately a 4-fold rise in the nucleotide 20 min after exposure to glucagon. The rather low sensitivity to cyclic 3′,5′-AMP added to the perfusion medium can presumably be ascribed to slow penetration into the cells and to rapid inactivation by its specific phosphodiesterase (2).

We interpret the changes in intermediates as follows: 1) The most consistent and usually largest change after stimulation of gluconeogenesis is a rise in phosphopyruvate relative to malate. This could be explained most easily by the activation of phosphopyruvate carboxylase. 2) The sharp fall in dihydroxyacetone phosphate relative to preceding intermediates suggests activation of dihydroxyacetone phosphate utilization. The patterns of intermediates suggest that the step activated is the conversion of fructose diphosphate to hexosemonophosphate in the liver from fed rats. In addition, a stimulation of glucose-6-phosphate to glucose may occur in the liver of fasted rats. The enzymes most likely to be concerned are fructose-diphosphatase and glucose-6-phosphatase. Hormonal regulation at this end of the pathway could be important for control of gluconeogenesis from glycerol. Nikkilä and Ojala (11) have suggested that glycerol is the major metabolic precursor of plasma glucose in the fasting rat. 3) In certain cases (*e.g.*, fig. 2), but not consistently, malate was elevated relative to pyruvate. This would suggest activation of pyruvate carboxylase under certain conditions. Localization of effects to these enzymes can only be tentative in view of the uncertainties involved in the interpretation of changes in the levels of intermediates. The changes, however, may at least delineate the metabolic regions which should be studied further.

The present results suggest that the list of systems affected by cyclic 3′,5′-

AMP may have to be expanded to include one or more enzymes in the pathway of gluconeogenesis.

SUMMARY

1. Gluconeogenesis from lactate in the perfused liver is stimulated by glucagon and the catecholamines. It is suggested that this effect has physiological significance.

2. The effect of the hormones on gluconeogenesis appears to be mediated by cyclic 3′,5′-AMP.

3. The hormones (or cyclic 3′,5′-AMP) activate more than one step in the pathway of lactate to glucose. The changes in the concentration of metabolic intermediates induced by the hormones are consistent with activations of phosphopyruvate carboxylase and the hexose phosphate phosphatases.

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