Effects of Glucagon on Glutamate Metabolism in the Perfused Rat Liver*

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SUMMARY

Early, rapid effects of glucagon on glutamate metabolism were studied in rat livers perfused with bicarbonate buffer containing bovine albumin and red cells, [14C]glutamate in tracer amounts, and physiological levels of lactate and pyruvate. Glucagon increased [14C]glucose synthesis from [1C]glutamate to a greater extent than from other labeled substrates. The effect was maximal at 3 min and was not inhibited by fluoroacetate.

The hormone decreased the tissue concentrations of glutamate, α-ketoglutarate, and citrate and increased those of alanine, aspartate, and P-pyruvate. In livers perfused with [14C]glutamate for 2 ½ min, glucagon increased the radioactivities of succinate, fumarate, aspartate, P-pyruvate, alanine, and glutamine, and decreased those of glutamate and α-ketoglutarate. Under these conditions, the specific radioactivities of aspartate, P-pyruvate, and alanine increased. These results are consistent with glucagon stimulation of the conversion of α-ketoglutarate to succinate, of the synthesis of P-pyruvate, and of the transamination of oxalacetate to aspartate and of pyruvate to alanine.

Tryptophan markedly inhibited the labeling of P-pyruvate and glucose in livers perfused with [14C]glutamate and increased the tissue levels of intermediates prior to P-pyruvate in the gluconeogenic pathway. Glucagon stimulation of [14C]glucose synthesis was abolished, but the hormone still produced changes in the levels and radioactivities of intermediates consistent with activation of a step between α-ketoglutarate and succinate. Arsenite produced changes in metabolite levels consistent with inhibition of α-ketoglutarate and pyruvate oxidation and abolished the effects of glucagon on [14C]glucose formation and on the levels and radioactivities of intermediates.

Glucagon stimulated the release of 14CO2 from [1-14C]glutamate. The effect was apparently unaltered by fluoroacetate but was abolished by arsenate. Tryptophan diminished the production of 14CO2 in control livers slightly but did not alter the stimulation by glucagon. These data provide further evidence of an effect of glucagon on α-ketoglutarate conversion to succinate and indicate that the effect is not dependent upon the stimulation of gluconeogenesis at another site.

In liver perfused with [14C]glutamate for 10 min, glucagon decreased the radioactivities of glutamate, ketoglutarate, malate, citrate, oxalacetate, and pyruvate and increased those of aspartate and P-pyruvate. The specific radioactivities of glutamate, α-ketoglutarate, malate, citrate, and aspartate were reduced by 50% or more. These data indicate that glucagon increases the production of unlabeled glutamate (presumably from endogenous protein); they also provide further evidence for a stimulation of P-pyruvate synthesis from oxalacetate.

Glucagon stimulates gluconeogenesis in the perfused rat liver (3–5), but the mechanism of the effect is incompletely understood. Evidence has been presented that an action of glucagon mediated by adenosine 3′:5′-monophosphate is exerted on a reaction(s) located between pyruvate and P-pyruvate in the gluconeogenic pathway (5, 6). During investigations designed to define further the way in which glucagon acts, it was observed that the hormone caused large changes in the metabolism of glutamate and α-ketoglutarate. This paper describes these changes and discusses their possible relationship to the gluconeogenic action of glucagon.

EXPERIMENTAL PROCEDURE

Animals—Male rats of the Sprague-Dawley strain maintained on Purina laboratory chow were used. Livers were taken from rats weighing 110 to 140 g fed ad libitum.

Technique of Liver Perfusion—The perfusion apparatus and procedure have been described (7). The perfusion medium consisted of oxygenated Krebs-Ringer bicarbonate buffer, bovine serum albumin, and bovine erythrocytes as described previously (7, 8). In all experiments livers were initially perfused for 1 hour with a recirculating medium. At the end of this period, sodium lactate and sodium pyruvate were added to the medium (100 ml) in the reservoir to produce 1 mM and 0.1 mM, respectively. Glucagon and 14C-labeled substrates, when present, were also added at this time. The perfusion was then changed to a nonrecirculating or "flow-through" system (7, 8) to maintain...
a constant substrate level allowing a close approach to a steady state.

Analysis of Medium—Previously described methods (7) were used to measure glucose and [14C]glucose in plasma from samples of effluent medium taken at intervals during flow-through perfusion.

With [14C]lactate as substrate, the procedure for [14C]glucose measurement (7) was modified by repeating the treatment with Dowex 50 and duolite resin. This was done because the error introduced by the small amount of [14C]lactate surviving a single resin treatment was significant. 14CO2 was measured by withdrawing 1 ml of perfusate in a syringe and injecting this into a rubber-stoppered vial equipped with a suspended center well (Kontes) containing 0.25 ml of Hyamine hydroxide. Perchloric acid (0.5 ml, 30%) was then injected into the perfusate and the vials were left overnight. 14CO2 absorbed into Hyamine was measured by scintillation spectrometry.

Extraction of Tissue—Livers were freeze-clamped at the end of perfusion and pulverized as described previously (6, 7). Tissue powder (2 g) was rapidly mixed with 2 ml of 30% HClO4 at 20° and the mixture was diluted 4-fold with ice-cold 4 mM EDTA. Protein-free supernatant obtained by centrifugation at 0° was titrated with 28% KOH to give a final pH of 3.5 to 4.0 and kept at ice temperature for 45 min to precipitate completely KClO4. Extracts so prepared were used immediately for separation of 14C compounds or stored (less than 1 week) at -15° for measurements of intermediates.

Enzymatic Analyses of Intermediates—Neutralized extracts were analyzed for alanine, pyruvate, malate, and P-pyruvate (7, 8), citrate (9), aspartate (10), and α-ketoglutarate (11). Glutamate was determined by first being converted to α-ketoglutarate by incubating 0.2 ml of neutralized extract with 2 μmoles of oxalacetate, 3 μmoles of NAD+, 9 units of glutamate dehydrogenase, and 0.75 unit of malate dehydrogenase in 0.5 ml of 0.15 M triethanolamine-HCl buffer (pH 7.6) for 30 min at 37°. The reaction was stopped with 0.1 ml of 60% HClO4, and the supernatant was neutralized with KOH before enzymatic analysis of α-ketoglutarate (11). Values were corrected for α-ketoglutarate originally present. Duplicates differed by less than 3% and recovery of 40 to 500 μmoles of added glutamate was 96 ± 1%.

Thin Layer Chromatography of Organic Acids—Portions (0.05 ml) of Fraction C were applied to plates of Silica Gel G, 0.25 mm thickness, and these were developed with water-saturated ether-HCOOH (5:1) containing 2%, 2,7’-dichlorofluorescein (13). Fumarate, succinate, malate, and citrate were identified by fluorescent spots under ultraviolet light by comparison with authentic samples.

Separation of Pyruvate, Oxalacetate, and α-Ketoglutarate—Because of their tendency to decompose during chromatography, α-ketoc acids were converted to their 2,4-dinitrophenyldiazones before separation. Tissue powder (0.2 g) was mixed with 0.2 ml of 30% HClO4 at -20° and then 0.2 ml of 0.2% 2,4-dinitrophenyldiazine in 2 N HCl was added, followed by 0.5 μmole of oxalacetate as carrier. Protein-free supernatant was neutralized with KOH at ice temperature, reacidified with 0.1 ml of 3 N HCl, and extracted three times with 2 ml of ethyl acetate. The combined ethyl acetate phases were re-extracted with 3 volumes of 2 ml of 1 N NH4OH. The NH4OH extracts containing the hydrazones of the keto acids were lyophilized, redissolved in 2 ml of 0.02 N NH4OH, acidified with HCl, and re-extracted three times with 2 ml of ethyl acetate. The combined ethyl acetate extracts were dried in a stream of air at room temperature. The dry residue was dissolved in 0.2 ml of 0.25% NH4OH and applied to 0.5-mm cellulose chromatoplates (Cellulose MN 300 HR, Macherey Nagel) in 0.05 ml volume. Plates were developed with 1-butanol-ethanol-0.5 N NH4OH (7:1:2) at 0°. The hydrazones of oxalacetate and α-ketoglutarate were well separated by this system and were not contaminated with other organic acids.

Separation of Labeled P-pyruvate—Neutralized HClO4 extracts (1 ml) were mixed with 1 ml of 0.15 N triethanolamine-HCl buffer (pH 7.6), 0.05 μmole of P pyruvate as carrier, 1 μmole of NADH, and 5 units of lactate dehydrogenase. The mixture was incubated at 37° for 10 min to convert pyruvate to lactate. Deproteinization with HClO4, and neutralization with KOH were performed as described under “Extraction of Tissue.” The KClO4 precipitate was washed with 1 ml of triethanolamine buffer and the washing combined with the supernatant. P-pyruvate in the mixture was converted to the 2,4-dinitrophenyldiazone of pyruvate by incubation with 3 μmoles of ADP, 5 units of pyruvate kinase, 100 mM KCl, and 10 mM MgCl2 for 15 min at 37° followed by 20 min with 0.2 ml of 0.2% 2,4-dinitrophenyldiazine in 2 N HCl. Extraction and chromatography of the hydrazones of pyruvate were performed as described above.

Determination of [14C]Glycogen—Fraction A obtained by column chromatography of neutralized extracts was lyophilized and dissolved in 2 ml of H2O. Four milliliters of ethanol and 0.1 ml of 84% Na2SO4 were added, the tube contents were brought to the boil, and the precipitated glycogen was centrifuged. Its chemical amount was determined by the anthrone method (14), and its radioactivity was measured by scintillation spectrometry.

Measurements of Radioactivity—Spots separated on chromatoplates were scraped off into counting vials containing 10 ml of seintillation fluid (5 g of 2,5-diphenyloxazole (PPO) and 0.3 g of 1,4 bis[2 (5 phenyloxazoyl)]benzene (POPPOP) per liter of toluene). Radioactivity was measured using a Packard Tri-Carb spectrometer. Quenching was corrected for by the use of an internal standard. It was negligible for the amino acids and organic acids but was substantial for the α-keto acid phenylhy-
drazones. The total radioactivity and specific radioactivity of compounds were corrected for losses during the separation procedures by determining the recovery of added standards. Recoveries ranged between 76 ± 2% for citrate and 90 ± 3% for malate.

Chemicals—Enzymes used for determinations were of analytical grade from Boehringer or Sigma. Coenzymes were from Sigma. Radioactive compounds were from Amersham-Searle and, where relevant, were L isomers. Glucagon was a kind gift from Lilly Research Laboratories.

RESULTS

Effects of Glucagon on [14C]Glucose from Various 14C-Labeled Substrates—In the first experiments, the early, rapid effect of glucagon on glucose production from several substrates was tested. Three of these substrates, lactate, pyruvate, and bicarbonate, enter the gluconeogenic pathway before or at the pyruvate carboxylase reaction, whereas glutamate and aspartate bypass this step, which has frequently been postulated to be a site of glucagon activation. Aspartate, in tracer levels, can presumably be incorporated into glucose without entering the mitochondria. Livers from fed rats were perfused for 1 hour with recirculating medium and then with nonrecirculating medium containing 1 mM lactate, 0.1 mM pyruvate, and trace amounts of various 14C-labeled precursors. The initial period of recirculation allows time for inactivation of glycogenolysis and presumably be incorporated into glucose without entering the mitochondria. The employment of nonrecirculating medium for the test period ensures known and steady levels of substrates and prevents recirculating of isotope.

Table I shows the effects of 5 × 10⁻⁹ M glucagon on the synthesis of [14C]glucose during short term (2½ hr min or less) exposure to the labeled substrates. The hormone increased the incorporation of isotope from labeled lactate, pyruvate, bicarbonate, and aspartate 2- to 4-fold. With [14C]glutamate as substrate, labeling of glucose in control livers was less, but the glucagon effect was greater (7-fold). Neither the basal rate of [14C]glucose synthesis from [14C]glutamate nor the effect of glucagon was significantly decreased by addition of fluorocacetate 15 min prior to substrate. The greater effect of glucagon on gluconeogenesis from glutamate than from aspartate suggested that the hormone may have a site(s) of action between glutamate and the 4-carbon dicarboxylic acids in addition to those located between the 4-carbon dicarboxylic acids and glucose. Glutamate may yield 4-carbon intermediates by transamination to α-ketoglutarate and forward operation of the citric acid cycle, or it may yield oxaloacetate by backward operation of the cycle coupled with citrate lyase action (15). The failure of fluorocacetate (which yields fluorocitrate, an inhibitor of aconitase and citrate lyase) to inhibit basal or glucagon-stimulated gluconeogenesis from glutamate suggested that the contribution of the second pathway was minimal.

Time Course of Gluconeogenesis from [14C]Glutamate—Livers were perfused with 1 mM lactate, 0.1 mM pyruvate, and [U-14C]glutamate in the absence or presence of glucagon. Fig. 1 shows the changes in [14C]glucose in the effluent medium when the labeled substrate was added at the start of the flow-through perfusion or 5 min later. It is seen that glucagon produced an almost immediate acceleration of gluconeogenesis. The steady rate of [14C]glucose synthesis in the presence of glucagon was about 5-fold higher than in control livers (Fig. 1 and other data not shown). The magnitude and the time course of the glucagon

![Figure 1](http://www.jbc.org/)

**Figure 1.** Time course of [14C]glucose production from [U-14C]glutamate in the presence or absence of glucagon. Basic conditions for perfusion and addition of lactate, pyruvate, and glucagon were the same as in Table I. L-[U-14C]Glutamate (2.43 × 10⁶ cpm per ml of perfusate) was added at the start of flow-through (○—○) or 5 min later (△—△). Vertical bars, 2 S.E.M.; figures in parentheses, number of observations.
Liver from fed rats perfused with \( \text{L-[U-}^{14}\text{C]} \text{glutamate} \) for 2.5 min as described in Table I were analyzed for the intermediates as indicated. Values are the means ± standard error of the mean with number of observations in parentheses. Alanine and glutamine were determined in combined samples from six control and six glucagon perfusions.

### Table II

<table>
<thead>
<tr>
<th>Chemical Amount</th>
<th>Radiactivity</th>
<th>Specific Radioactivity</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Glucagon</td>
</tr>
<tr>
<td>Glutamate</td>
<td>2353 ± 53 (14)</td>
<td>76 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>314 ± 14 (57)</td>
<td>35 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Succinate</td>
<td>54 ± 5 (11)</td>
<td>290 ± 15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fumarate</td>
<td>148 ± 6 (41)</td>
<td>100 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Malate</td>
<td>525 ± 10 (40)</td>
<td>138 ± 6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>550 ± 8 (27)</td>
<td>89 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aspartate</td>
<td>98 ± 5 (30)</td>
<td>171 ± 8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P-pyruvate</td>
<td>758 ± 6 (164)</td>
<td>757 ± 170&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup> p < 0.01.
<sup>b</sup> p < 0.05.

Changes in those of other compounds measured. Although these data cannot be interpreted unequivocally because of potential problems relating to compartmentation, they are consistent with glucagon stimulation of the Krebs cycle at a site located between α-ketoglutarate and succinate.

**Effects of Metabolic Inhibitors on Distribution of Radioactivity in Intermediates in Livers Perfused with \( \text{[U-}^{14}\text{C]} \text{Glutamate} \)**—To obtain further evidence of the effect of glucagon on the citric acid cycle suggested above, livers were perfused under the conditions described in Table II in the presence of several inhibitors. Fig. 3 shows that tryptophan, an inhibitor of P-enolpyruvate carboxykinase (16), increased the levels of intermediates prior to P-pyruvate in the gluconeogenic pathway. The agent also reduced the radioactivity of P-pyruvate by 90% and that of glucose by 96% (Fig. 4). The labeling of intermediates prior to P-pyruvate was generally increased (Fig. 4). In the presence of tryptophan, the glucagon stimulation of the incorporation of isotope from \( \text{[U-}^{14}\text{C]} \text{glutamate} \) into glucose was abolished (Fig. 5). Under these circumstances, glucagon did not increase the labeling of P-pyruvate, decreased that of α-ketoglutarate, and increased that of the 4-carbon dicarboxylic acids (Fig. 5). The changes produced by glucagon in the chemical amounts of intermediates were similar to those in radioactivity with decreases in glutamate and α-ketoglutarate, and increases in malate and aspartate (Fig. 6). However, P-pyruvate was increased slightly, suggesting that glycolysis may have been increased consequent to enhanced gluconeogenesis.

Arsenite, an inhibitor of α-keto acid dehydrogenases, produced changes in the levels of intermediates in livers perfused with lactate, pyruvate, and \( \text{[U-}^{14}\text{C]} \text{glutamate} \) which were consistent with inhibition of α-ketoglutarate and pyruvate oxidation. There were large rises in α-ketoglutarate and pyruvate, and falls in malate and citrate (Fig. 7). Arsenite essentially abolished the effects of glucagon on \( \text{[U-}^{14}\text{C]} \text{glucose} \) formation and on the radioactivities and levels of metabolic intermediates (Figs. 5 and 6). Fluoracetate, an inhibitor of aconitase and citrate lyase, caused an increase in citrate in livers perfused as described above.

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1. The figure presents key changes in the levels and radioactivities of intermediates produced by glucagon so that they may be compared more readily with those seen in the presence of metabolic inhibitors (Figs. 5 and 6).

2. The specific radioactivity of glutamate given in Tables II and III is the average value for the tissue, i.e. extracellular and intracellular glutamate. If it is assumed that 1 g of liver contains 0.2 ml of extracellular fluid (8) and that the \( \text{[U-}^{14}\text{C]} \text{glutamate} \) in the initial perfusate is distributed uniformly in the extracellular fluid, calculations from the data of Table II indicate that the average specific radioactivity of intracellular glutamate was 46 cpm per nmole, i.e. considerably less than that of \( \text{L-[U-}^{14}\text{C]} \text{glutamate} \), its immediate product. As will be discussed, this is probably due to a transient lack of equilibrium of isotope between two or more intracellular pools of glutamate. The discrepancy between the specific radioactivity of glutamate and its products is not evident in experiments in which the exposure to \( \text{[U-}^{14}\text{C]} \text{glutamate} \) was longer (Table III).
FIG. 2. Effects of glucagon on the % content and concentration of intermediates in livers perfused with [U-14C]glutamate. Data are from Table II. A shows radioactivity and B shows chemical amount.

(Fig. 3). It was virtually without effect on the changes induced by glucagon in the radioactivities and levels of intermediates (Figs. 5 and 6, cf. Fig. 2). These data suggest that neither citrate conversion to isocitrate nor citrate cleavage to oxalacetate are involved in the stimulatory effect of gluconeogenesis from glutamate.

Stimulation of α-Ketoglutarate Decarboxylation by Glucagon—To obtain further evidence for a stimulating effect of glucagon on the conversion of α-ketoglutarate to succinate, livers were perfused with [1-14C]glutamate and the effects of glucagon on 14CO2 examined. Fig. 7 shows that 14CO2 production in the absence of hormone rose rapidly to reach a steady rate at about 12 min. Glucagon increased the rate of 14CO2 release 5-fold at 3 min. Thereafter the glucagon effect diminished and was largely lost at 12 min. As was noted in the studies of the time course of 14Cglucose synthesis, the magnitude and the time course of the glucagon effect were virtually unaltered by delaying addition of [14C]glutamate until 7 min.

To identify further the site of glucagon action on α-ketoglutarate decarboxylation, perfusions were carried out in the presence of fluoroacetate and in the presence or absence of arsenate. Addition of fluoroacetate alone apparently produced little change in the time course of 14CO2 production from [1-14C]glutamate in control livers (Fig. 8, cf. Fig. 7), taking into account the difference in time of addition of labeled substrate, and did not abolish the effect of glucagon on 14CO2 release. Two minutes after glucagon addition, 14CO2 production was increased 2-fold, but thereafter the effect diminished (Fig. 8). Addition of arsenate with fluoroacetate did not significantly alter 14CO2 formation in control livers but greatly reduced the stimulation by glucagon (Fig. 8). The inhibitory effect of arsenate, an agent which uncouples substrate level and oxidative phosphorylation, suggests that the action of glucagon on α-ketoglutarate decarboxylation is dependent on the production of ATP or GTP.

Addition of tryptophan to the perfusion medium 45 min prior to flow-through with [1-14C]glutamate diminished the control rate of 14CO2 release by about 30% (Fig. 9). The magnitude and the time course of the glucagon effect were essentially the same as in livers perfused without tryptophan. These data are consistent with the view that the stimulatory effect of glucagon on α-keto-
Effects of glucagon on the α-KG content of intermediates in livers perfused with [U-C^14]glutamate in the presence of inhibitors plotted as percentages of values without glucagon. The experimental conditions were the same as in Fig. 2. Glucagon (5 × 10^{-9} M) and [U-^{14}C]glutamate (4.37 × 10^{6} cpm) were infused for 25 min. α KG, α-ketoglutarate; Succ, succinate; Fum, fumarate; Mal, malate; Asp, aspartate; Cit, citrate; OAA, oxalacetate; PEP, P-pyruvate.

Glutamate and α-Ketoglutarate in Livers Perfused for 10 Min with [U-^{14}C]Glutamate—The transient nature of the glucagon stimulation of 14CO2 release from [L-^{14}C]glutamate suggested that glucagon may also be causing a slow reduction in the specific radioactivity of [α-^{14}C]glutamate or α-keto[α-^{14}C]glutamate, or both. To examine this point, samples of livers were rapidly frozen after perfusion for 10 min with [α-^{14}C]glutamate in the absence or presence of glucagon. Table III shows the chemical amounts and specific radioactivities of glutamate and α-ketoglutarate determined in these livers. It is seen that glucagon not only brought about a marked decline in the chemical level of glutamate and α-ketoglutarate but also a disproportionately greater fall in radioactivity. Since the specific activity of glutamate fell almost as much as that of α-ketoglutarate, it is probable that unlabeled glutamate was the main source of the carbon diluting the labeled α-ketoglutarate. Some additional dilution of α-ketoglutarate, however, presumably occurred as a consequence of a faster decarboxylation of isocitrate since glucagon can cause a modest increase in Krebs cycle activity (17). The source of unlabeled glutamate was presumably hepatic protein, the breakdown of which is known to be stimulated by glucagon (8).

Labeling of Metabolic Intermediates after Longer Exposure to [1-^{14}C]Glutamate—In livers perfused for 10 min with [U-^{14}C]glutamate, glucagon produced alterations in metabolite levels which were not significantly different from those seen at 25 min except for a greater decrease in glutamate. The changes in radioactivity, however, were very different. There were marked decreases in the labeling of glutamate, α-ketoglutarate, malate, oxalacetate, citrate, and pyruvate. The increases in succinate, fumarate, and aspartate radioactivities largely disappeared, while those of P-pyruvate, alanine, and glutamine were little altered (Table III).

The relative specific radioactivities of metabolites in control livers were consistent with a pathway of P-pyruvate synthesis from glutamate via α-ketoglutarate formation and forward operation of the Krebs cycle. Glucagon induced substantial decreases in the specific radioactivities of glutamate, α-ketoglutarate, malate, citrate, and pyruvate. The increases in succinate, fumarate, and aspartate radioactivities largely disappeared, while those of P-pyruvate, alanine, and glutamine were little altered (Table III).

In summary, the observations at 10 min support the view that glucagon produced multiple effects on glutamate metabolism in these experiments: (a) stimulation of the formation of unlabeled glutamate and, perhaps, α-ketoglutarate; (b) activation of α-ketoglutarate oxidation to succinate; (c) activation of P-pyruvate synthesis from 4-carbon dicarboxylic acids; (d) stimulation of alanine and glutamine formation.

DISCUSSION

The conclusion that glucagon induces a rapid activation of the oxidative decarboxylation of α-ketoglutarate in the liver is sup-
ported by the following observations: (a) the increased release of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$glutamate which is abolished by arsenate and arsenite, but not by fluoroacetate; (b) the increased labeling of succinate and fumarate and decreased labeling of $\alpha$-ketoglutarate in livers perfused with $[U-^{14}\text{C}]$glutamate; (c) the marked decrease in $\alpha$-ketoglutarate concentration in the face of an unchanged level of malate and an increased level of aspartate.

Under the conditions of these experiments, i.e., with physiological levels of lactate plus pyruvate, glucagon appears to stimulate the Krebs cycle slightly (17). This has also been reported to occur in livers perfused without exogenous substrate (18). The present findings suggest that the effect may be partly due to acceleration of $\alpha$-ketoglutarate oxidation. In addition, an increased rate of this reaction could help explain glucagon stimulation of endogenous gluconeogenesis and ureogenesis in the liver since increased utilization of glutamate by way of the citric acid cycle could generate aspartate for glucose and urea formation (see Fig. 10).

3 Since glucagon decreased the specific radioactivity of $\alpha$-ketoglutarate, the increase in decarboxylation was probably more marked than indicated by the change in radioactivity.

Fig. 8. Effect of glucagon on $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$glutamate in the presence of fluoroacetate and in the presence or absence of arsenate. Livers from fed rats were perfused with glucagon ($5 \times 10^{-9} \text{M}$) in a flow-through system after 1 hour of initial perfusion with recirculating medium as described in Table I. Sodium fluoroacetate, 1 mM, and $[1-^{14}\text{C}]$glutamate, $4.3 \times 10^6 \text{cpm}$ per 80 ml of medium, were added into the recirculating medium 15 min and 5 min prior to the start of flow-through, respectively. Lactate (1 mM) and pyruvate (0.1 mM) were added together with the isotopic compound. Sodium arsenate, when present, was added 15 min prior to glucagon. Each point represents the mean value for the $^{14}\text{CO}_2$ content of 1 ml of perfusate leaving the liver at the time indicated. Three livers were perfused in each category. Values for glucagon in the absence of arsenate were significantly different from control values at 0.5 min and beyond. Differences between glucagon and control values in the presence of arsenate were not significant. Differences between control values in the absence and presence of arsenate were not significant.

Fig. 9. Effect of glucagon on $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$glutamate in the presence or absence of tryptophan. Livers were perfused as described in Fig. 8 except that fluoroacetate was absent and $[1-^{14}\text{C}]$glutamate was continuously infused at a rate of $3.6 \times 10^6 \text{cpm}$ per min from the start of flow-through perfusion. Tryptophan, when present, was added 45 min prior to the start of flow-through perfusion to give a final concentration of 2.4 mM. Mean values for $^{14}\text{CO}_2$ content of 1 ml of perfusate leaving the liver were plotted with 2 S.E.M. as vertical bars. Numbers of observations were three for each plot.

The slight decrease in citrate induced by glucagon in the present study probably resulted from increased citrate utilization due to the activation of $\alpha$-ketoglutarate oxidation, although citrate synthesis may also have been diminished if mitochondrial oxaloacetate fell. Since an increase in gluconeogenesis would require an increase in ATP generation, the latter explanation seems unlikely since it would result in slowing of the Krebs cycle. Smith and Williamson (19) have recently shown that succinyl-CoA inhibits citrate synthase. Changes in the level of this ester induced by glucagon could thus play a role in the regulation of the Krebs cycle.

The reactions between $\alpha$-ketoglutarate and succinate are catalyzed by the $\alpha$-ketoglutarate dehydrogenase complex and succinate thiokinase. The activity of the latter enzyme is coupled to GTP production from GDP, and arsenate acts as an uncoupler of the reaction. Failure of arsenate to increase $\alpha$-ketoglutarate decarboxylation in the present study (Fig. 8) suggests that either the decarboxylation reaction was not limited by succinyl-CoA removal or that the thiokinase reaction was not controlled by GDP supply. With these considerations in mind, the abolition by arsenate of the effect of glucagon on $\alpha$-ketoglutarate decarboxylation is not readily understood, except in terms of a general effect of arsenate on oxidative phosphorylation rather than a specific effect on succinate thiokinase. A dependence of the glucagon effect on the maintenance of ATP levels would be expected among other reasons if glucagon acted by a mechanism involving an adenosine 3':5'-monophosphate-dependent protein kinase.

It appears that the effect of glucagon on $\alpha$-ketoglutarate decarboxylation is not secondary to a stimulation of gluconeogenesis at the P-enolpyruvate carboxykinase step. This is shown by the fact that the stimulation of decarboxylation persists when gluconeogenesis is blocked by tryptophan. The question of whether the effect of glucagon at the carboxykinase level is
Table III
Effects of glucagon on tissue levels, 14C content, and specific radioactivity of intermediates in livers perfused with [U-14C]glutamate for 10 min.

Livers from fed rats were perfused under the same condition as in Table I except that the liver was excised and analyzed after 10 min of flow-through perfusion. Values are the means of six observations with standard error of the mean. Radioactive glutamate is the total value in tissue. The total radioactivity of [14C]glutamate infused was 9.28 × 10^6 cpm.

<table>
<thead>
<tr>
<th>Chemical amount</th>
<th>Control</th>
<th>Glucagon</th>
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<tbody>
<tr>
<td>Glutamate</td>
<td>3418 ± 182</td>
<td>37 ± 3a</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>415 ± 25</td>
<td>38 ± 4a</td>
</tr>
<tr>
<td>Succinate</td>
<td>78 ± 5</td>
<td>94 ± 2</td>
</tr>
<tr>
<td>Fumarate</td>
<td>11 ± 2</td>
<td>90 ± 7</td>
</tr>
<tr>
<td>Malate</td>
<td>130 ± 9</td>
<td>59 ± 4b</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>3.4 ± 0.6</td>
<td>41 ± 15</td>
</tr>
<tr>
<td>Aspartate</td>
<td>267 ± 23</td>
<td>135 ± 9b</td>
</tr>
<tr>
<td>Citrate</td>
<td>290 ± 16</td>
<td>49 ± 2b</td>
</tr>
<tr>
<td>P-pyruvate</td>
<td>8 ± 1</td>
<td>238 ± 18b</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>5 ± 0.4</td>
<td>31 ± 8b</td>
</tr>
<tr>
<td>Alanine</td>
<td>129</td>
<td>244</td>
</tr>
<tr>
<td>Glutamine</td>
<td>57 ± 2</td>
<td>118 ± 7</td>
</tr>
</tbody>
</table>

a p < 0.01.

Fig. 10. Scheme depicting postulated effects of glucagon on glutamate metabolism. Thick arrows indicate increases in reaction rates. Wavy lines indicate mitochondrial membranes. For the sake of clarity, movements of α-ketoglutarate or glutamate across the mitochondrial membranes associated with transamination have been omitted. Abbreviations not defined in Figs. 3 and 5 are: SUC-CoA, succinyl-CoA; ALA, alanine.

dependent upon the action of α-ketoglutarate decarboxylation is, on the other hand, unresolved. Both arsenate and arsenate abolished the action of the hormone on gluconeogenesis from lactate-pyruvate, but this could have been due to diminished ATP generation. A potential link between the two effects is abolished the action of the hormone on gluconeogenesis from oxalacetate or aspartate and of pyruvate to alanine. The stimulatory effect of glucagon on glutamine labeling could indicate an effect on glutamine synthetase. However, this is uncertain because of the likelihood that glutamate exists in two or more pools in liver (23) only one of which serves as a precursor of glutamine. The existence of more than one pool is indicated by the following observations. (a) The ratio of glutamine radioactivity to glutamate radioactivity was much higher in livers perfused with [14C]glutamate than in those perfused with H14CO3 or [14C]pyruvate (25). (b) Glucagon increased the labeling of glutamine in livers perfused with [14C]glutamate but not with the other substrates. (c) The specific

4 Regulation of P-enolpyruvate carboxykinase by changes in the supply of GTP seems a priori unlikely. As a general rule, metabolic control mechanisms which depend primarily on an increase in the level of ATP or other nucleotide triphosphates (i.e., push mechanisms) rather than on changes in the levels of "energy-rich" nucleotides (i.e., pull mechanisms) are unusual and teleologically undesirable. This is because the cell's capacity to generate ATP is seldom compromised and ATP levels do not fall except under extreme circumstances, e.g., during prolonged anoxia. Control is usually exerted at the sites of energy utilization.
radioactivities of α-keto[14C]glutarate and [14C]malate were higher than that of [14C]glutamate in short term perfusions with [14C]glutamate (Table II). The uncertainty about the specific activity of the glutamate pool from which glutamine was synthesized thus precludes identification of glutamine synthesis as a site of action of glucagon.

The reduction in α-ketoglutarate specific activity caused by glucagon in livers perfused with [14C]glutamate appears to be largely due to increased formation of unlabeled glutamate from endogenous protein. Glucagon is known to have a proteolytic action in liver and the dilution of label in glutamate is about the same as that in α-ketoglutarate as steady state conditions are approached (Table III). This would be in accord with a precursor-product relationship between glutamate and α-ketoglutarate. As noted earlier, some additional unlabeled α-ketoglutarate is probably formed from isocitrate secondary to acceleration of the Krebs cycle. Despite the increased input of unlabeled glutamate from protein catabolism, the tissue and perfusate levels of this amino acid decrease in livers perfused with glucagon, indicating a predominance of the hormone effect on glutamate and α-ketoglutarate utilization (24).

Glucagon stimulates the uptake of several amino acids in the liver (24), but no evidence was obtained in the present study for an effect of the hormone on glutamate transport. However, such an effect could have been masked by the increase in the intracellular utilization of the amino acid. The much smaller decrease in the radioactivity of glutamate relative to that of α-ketoglutarate in livers perfused with [14C]glutamate (Table II) suggests the occurrence of a transport effect. However, this may also be explained by the existence of a large pool of labeled glutamate which is not linked to the citric acid cycle.

Fig. 10 illustrates how some of the changes apparently induced by glucagon in the liver might be explained. It depicts a site of glucagon action on either α-ketoglutarate dehydrogenase or succinate thiokinase. The increased utilization of α-ketoglutarate leads to an increase in its formation by both glutamate dehydrogenase and glutamate-oxalacetate transaminase. Increased glutamate dehydrogenase produces NH₃ for the ornithine transcarbamylase reaction of the urea cycle. The increased utilization of α-ketoglutarate in the Krebs cycle leads to increased formation of oxalacetate which is transaminated to aspartate because of the increased glutamate-oxalacetate reaction. Aspartate leaves the mitochondrion and is converted to oxalacetate by way of fumarate in the urea cycle or by transamination with α-keto-glutarate. The oxalacetate is then converted to pyruvate and eventually to glucose. The over-all reaction would be the following.

\[
2\text{Glutamate} + 4\text{O}_2 \rightarrow 2\text{oxalacetate} + \text{urea} + \text{CO}_2 + 3\text{H}_2\text{O}
\]

An increase in malate efflux from the mitochondrion would transfer hydrogen into the cytosol and decrease aspartate production and hence transamination of glutamate to α-ketoglutarate. If glutamate oxidation were increased to make up the deficit in α-ketoglutarate production, there would be increased production of NH₃. This could increase the rate of the urea cycle causing increased consumption of aspartate in the cytosol and hence increased glutamate utilization. Since the data in the present study do not indicate the occurrence of these changes, they have been omitted from the figure.

Transamination would utilize α-ketoglutarate and form glutamate in the cytosol. This would consume some of the α-keto-glutarate generated in the mitochondrion and hence diminish gluconeogenesis from glutamate. However, it appears that α-ketoglutarate is partly regenerated in the cytosol by transamination of glutamate with pyruvate to form alanine.

Several corollaries can be drawn from this scheme because of the stoichiometric relationships between gluconeogenesis, ureogenesis, and the Krebs cycle. The first is that operation of the scheme would require that glucagon increase both glutamate oxidation and glutamate transamination (because of the need of the urea cycle for both NH₃ and aspartate). The second is that any increase in the formation of oxalacetate from aspartate via fumarate in the urea cycle would require increases in the glutamate dehydrogenase reaction, in the citric acid cycle, and in the conversion of oxalacetate to aspartate in the mitochondrion.

The scheme utilizes tissue glutamate which can be replenished by protein breakdown, or, in the in vivo situation, by glutamate uptake from blood. It can account for the enhancement of endogenous gluconeogenesis and ureogenesis by glucagon, but can only partly explain the effect of the hormone on glucose synthesis from lactate pyruvate or alanine. This action of glucagon is discussed in the following paper (25).

Finally, it should be noted that, although 14C was incorporated into glucose in these experiments, the degree of labeling was very small (less than 1% of the added [14C]glutamate). This is consistent with the findings of Krebs and co-workers (26, 27) that the rate of gluconeogenesis from exogenous glutamate in the perfused rat liver is very low due to the restricted permeability of the plasma membrane to this substrate. It is probable that the extremely low rate of metabolism of exogenous glutamate and the use of livers from fasted rats for perfusion accounts for the failure of Ross et al. (20) to observe a significant effect of glucagon on the product of glucose from glutamate. Although gluconeogenesis from extracellular glutamate appears to be of negligible quantitative significance, glucose synthesis from endogenously derived glutamate may contribute significantly to gluconeogenesis in livers perfused without added substrate.

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