Studies on the Perfused Rat Liver

II. Effect of Glucagon on Gluconeogenesis

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SUMMARY

In vitro studies using the isolated perfused liver from fasted rats have demonstrated increased glucose production in the presence of glucagon. The incorporation of C-14-labeled alanine into glucose was increased, as was production of urea, suggesting a primary effect of glucagon in stimulating gluconeogenesis. Time studies showed this effect to occur in ten to fifteen minutes. Incorporation of pyruvate and lactate C-14 was also increased, as was that of HC-14-O_3. DIABETES *15*:188-93, March, 1966.

The hyperglycemia produced by glucagon is attributed mainly to glycogenolysis.[1] It has been shown that glucagon stimulates production of cyclic 3'-5' adenosine monophosphate, the cofactor that leads to the formation of active phosphorylase.[2] Other effects of glucagon have been demonstrated which suggest that this hormone may stimulate glucose production through pathways other than glycogenolysis. Miller[3] has shown in the isolated perfused rat liver that glucagon results in increased urea production. Glucagon has been shown to promote increased protein catabolism with decreased blood amino acid concentration, increased hepatic uptake of alpha amino nitrogen and increased nitrogen excretion.[4-6] Its effect on ketone body production is not well established.[1]

In the preceding paper[7] it was suggested that in the isolated perfused liver of the fasted rat, glucagon increases production concomitantly with urea production. This report deals with studies designed to examine the effects of glucagon on synthesis of glucose from precursors other than glycogen.

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MATERIAL AND METHODS

Liver perfusion. The perfusion technic, including apparatus, perfusion medium, dietary condition and weight of rats has been described.[7] In the present studies, however, perfusion was carried out for only two hours following administration of glucagon or control solution.

Substrates. Glucose, 1-alanine, fructose, galactose, pyruvate and lactate were all at 10 mM concentration unless otherwise indicated. Uniformly labeled 1-alanine-C-14, specific activity 123 mc./mM; sodium pyruvate-2-C-14, specific activity 3.06 mc./mM; sodium lactate-1-C-14, specific activity 5.47 mc./mM; and sodium bicarbonate-C-14, specific activity 18.6 mc./mM were obtained from the New England Nuclear Corp. Ten microcuries of each were used per perfusion. Glucagon solution was prepared from crystalline glucagon, Lot No. 258-234B-167-1, kindly supplied by Dr. W. R. Kirtley of the Lilly Laboratories. A single dose of 200 μg. per liver was administered.

Analyses. The determination of glycogen, medium glucose and urea was as described.[7] Fructose was determined by the resorcinol reaction method of Higashi and Peters.[8]

Isotopic. Glucose specific activity was determined as follows. Medium glucose was deproteinized by the method of Somogyi.[9] Glucose carrier was added to the clear filtrate and glucose osazones were prepared; the latter were recrystallized with ethanol, washed, plated on stainless steel planchets[10] and counted in a gas flow counter.[11]

C-14 activity in glycogen, in medium and in evolved CO_2 was determined as in the preceding report.[7] Total radioactivity in glucose, glycogen and CO_2, expressed as dpm/gm. wet liver, was determined. From this and the total radioactivity of each label added, the incorporation of label into glucose, glycogen and CO_2 was calculated and expressed as per cent.

RESULTS

Hyperglycemia and urea production

Figure 1 shows the results of three sets of perfusions in which medium glucose and urea were determined with time. Perfusion medium was without substrate other than glucose (10 mM). It is seen that in control livers there is only a slight production of urea while glucose utilization predominates, as indicated by a falling medium glucose concentration. In contrast, with glucagon there is a much greater increment in urea production which at the end of two hours is about twice that of controls. There is also a continuous increase in medium glucose indicating glucose production exceeding glucose utilization.

Perfusion with l-alanine-C-14

In order to determine whether the simultaneous increase in glucose and urea production with glucagon reflects increased amino acid utilization for glucose synthesis, livers were perfused with medium containing uniformly labeled l-alanine-C-14. It is evident from figure 2 that glucagon stimulates the conversion of alanine into glucose as shown by an increase in medium glucose specific activity over that of controls. The effect attains a peak in about forty minutes and plateaus thereafter.

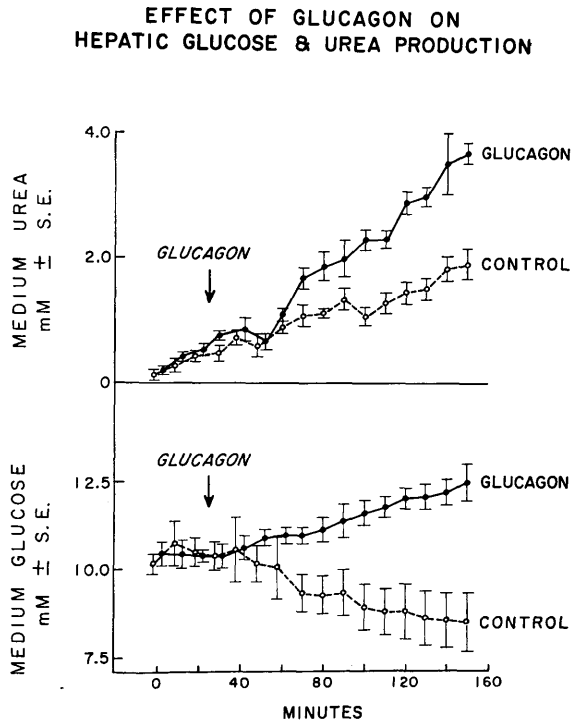

FIG. 1. Mean values with standard errors in brackets for six perfusions with or without the addition of glucagon (200 μg.) at thirty minutes. By fifteen minutes there appears to be glucose production by those given the hormone whereas the control livers show a net utilization of glucose. There is approximately a 2:1 ratio in the difference in moles of glucose to moles of urea between glucagon-exposed and controls.

Perfusion with l-alanine-C-14 and carrier alanine

Figure 3 illustrates the results of six sets of perfusions at three concentrations of l-alanine: trace quantities (approximately 0.001 mM), 10 mM and 50 mM alanine. All contained the same amount of l-alanine-C-14. At trace quantities of alanine, glucagon results in a definite increase in alanine-C-14 incorporation into glucose. Incorporation into glycogen on the other hand is significantly reduced. Glucagon results in a net increase of perfusate glucose compared to a decrease in controls. Urea production is increased to twice that of controls. Glycogenolysis occurs, but the amount of glycogen breakdown (—4.1 ± 2.0 μmoles/gm.) cannot account for the increase in perfusate glucose (+ 24 ± 4 μmoles/gm.).

At 10 mM alanine concentration, glucagon again stimulates incorporation of alanine-C-14 into glucose and decreases that into glycogen; no difference is seen in C-14 incorporation into CO_2. Net glucose production is increased greater than that at 0.001 mM alanine. There is also a marked increase in urea production which is 50 per cent greater than that seen at 0.001 mM alanine. Glycogenolysis is inhibited in controls but still occurs with glucagon; again the amount of glycogen metabolized (—5.2 ± 2.1 μmoles/gm.) cannot account for the net increase in medium glucose (+ 37 ± 5 μmoles/gm.). With 50 mM alanine, all

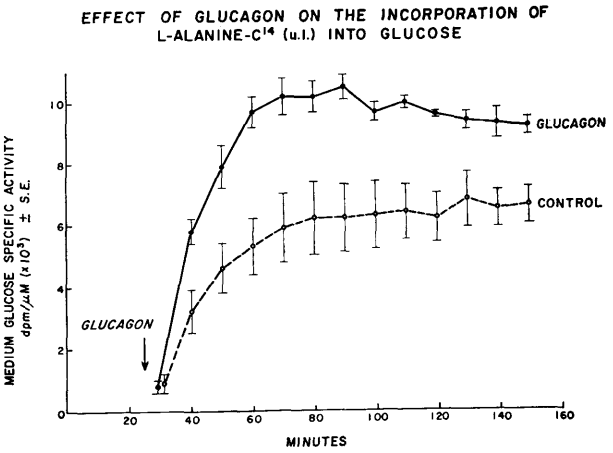

FIG. 2. Sequential incorporation of l-alanine-C-14 (trace dose) into perfusate glucose following administration of glucagon to the perfused liver. Standard errors are bracketed, mean of six experiments in treated and untreated groups.

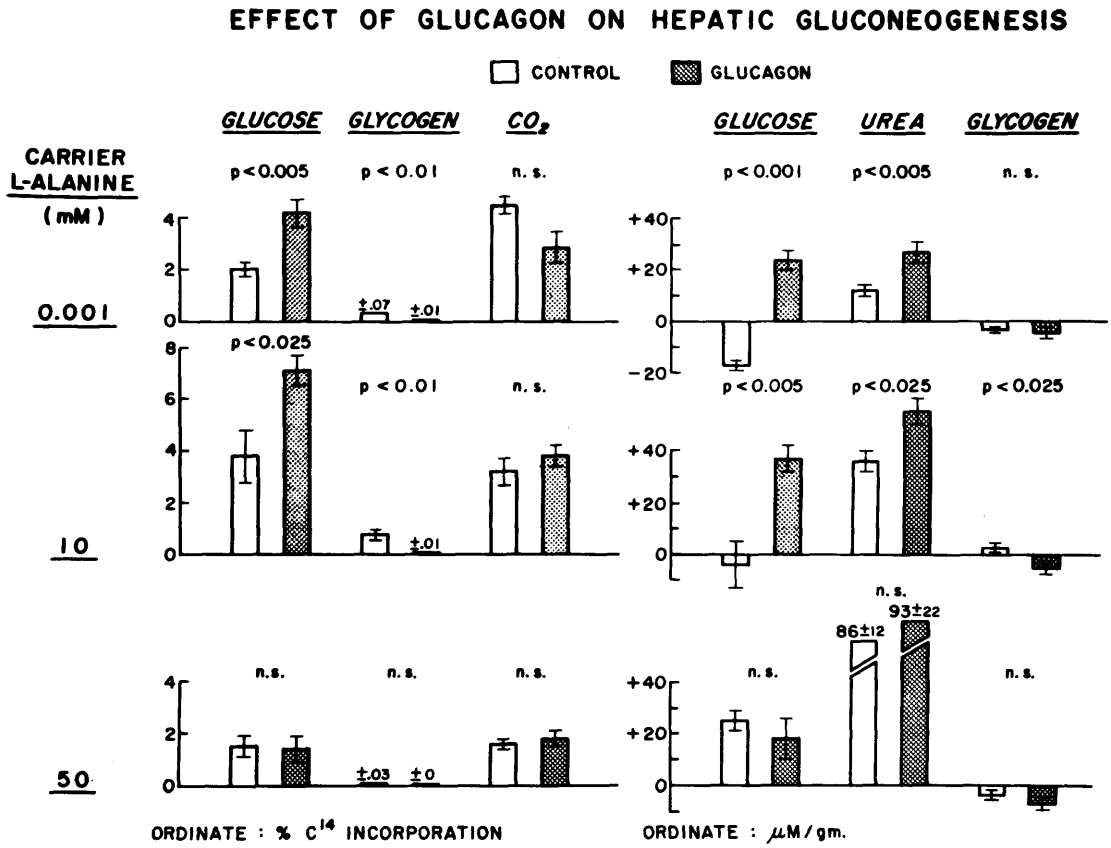

FIG. 3. Incorporation of l-alanine-C-14 in trace amounts, 10 mM and 50 mM, into glucose, glycogen and CO_2 by perfused rat livers drawn on the left of the figure and net changes in medium glucose, urea and liver glycogen drawn on the right. All values are expressed per gram of wet liver for a two-hour perfusion period.

parameters are reduced, probably due to a nonspecific toxic effect of this high concentration of amino acid.

Perfusion with pyruvate-2-C-14 or lactate-1-C-14

Since alanine is utilized for hexose synthesis through transamination to pyruvic acid, it was of interest to determine whether glucagon would stimulate conversion of pyruvate or lactate into glucose. Table 1 shows the results of perfusions in the presence of pyruvate-2-C-14 or lactate-1-C-14. In the presence of either substrate, glucagon results in a net increase of medium glucose which is significant in this limited number of experiments with pyruvate and not with lactate. There is glycogenolysis but, again, to a degree lesser than the rise in medium glucose. Glucagon stimulates incorporation of C-14 label from both substrates into glucose and decreases incorporation into glycogen. It has no effect on incorporation into CO_2.

Perfusion with bicarbonate-C-14

Table 2 shows the results of perfusions with pyruvate or lactate in the presence of bicarbonate-C-14. With either substrate glucagon again leads to a highly significant increase in perfusate glucose. Although glucagon results in glycogenolysis, the changes are no different from those in controls and the values are much lower than the increases in perfusate glucose. Incorporation of bicarbonate-C-14 into glucose is increased by glucagon and that into glycogen is significantly reduced.

Perfusion with fructose or galactose

In table 3 are shown the results of perfusions in the presence of either fructose or galactose. It is surprising that glucagon results in marked increases in medium glucose in the presence of either substrate. Glycogenolysis with glucagon is no different from that with controls and is again negligible when compared to the rise in medium glucose. There is no effect of glucagon on fructose disappearance. That of galactose was not determined.

TABLE 1
Effect of glucagon on metabolism of pyruvate-2-C-14 and lactate-1-C-14 by the perfused rat liver

	Δ Glucose (μM/gm.)	Δ Glycogen (μM/gm.)	C-14 incorporation into Glucose* (per cent)	Glycogen* (per cent)	CO_2* (per cent)
Pyruvate-2-C-14, no carrier (3)†					
Control	−17±2	−0.58±6.76	0.80±0.22	1.93±0.35	3.85±0.59
Glucagon	+16±1	−7.42±2.30	6.02±1.3	0.05±0.005	3.61±0.7
P	<0.001	not significant	<0.025	<0.005	not significant
Lactate-1-C-14, no carrier (3)†					
Control	+16±22	+1.11±1.88	1.02±0.13	0.66±0.2	9.42±1.88
Glucagon	+29±11	−2.10±0.82	2.54±0.39	0.02±0.001	9.19±1.76
P	not significant	not significant	<0.025	<0.05	not significant

*Per cent of total dpm/gm. over total dpm dose.
†Numbers in parenthesis indicate numbers of perfusion.
Glucose 10mM. Values with S.E.M.

TABLE 2
Effect of glucagon on metabolism of pyruvate and lactate in the presence of bicarbonate-C-14 by the perfused rat liver

	Δ Glucose (μM/gm.)	Δ Glycogen (μM/gm.)	C-14 incorporation into Glucose* (per cent)	Glycogen* (per cent)
Pyruvate, 10 mM and bicarbonate-C-14 (3)†				
Control	+ 3 ± 1	+2.47 ± 1.7	0.31 ± 0.04	0.033 ± 0.013
Glucagon	+43 ± 3	−3.59 ± 1.06	0.53 ± 0.1	0.005 ± 0
P	<0.001	not significant	not significant	<0.05
Lactate, 10 mM and bicarbonate-C-14 (3)†				
Control	+ 8 ± 3	+0.83 ± 2.18	0.27 ± 0.07	0.042 ± 0.012
Glucagon	+74 ± 11	−4.94 ± 1.94	0.74 ± 0.05	0.006 ± 0
P	<0.005	not significant	<0.01	<0.025

*Per cent of total dpm/gm. over total dpm dose.
†Numbers in parentheses indicate numbers of perfusions.
Glucose 10 mM. Values with S.E.M.

TABLE 3
Effect of glucagon on metabolism of fructose and galactose by the perfused rat liver

	Δ Glucose (μM/gm.)	Δ Glycogen (μM/gm.)	Δ Fructose (μM/gm.)
Fructose, 10 mM (6)*			
Control	+ 3 ± 6	+1.11 ± 2.12	−29 ± 9
Glucagon	+58 ± 10	−2.12 ± 0.83	−29 ± 9
P	<0.001	not significant	
Galactose, 10 mM (3)*			
Control	+ 2 ± 5	+2.79 ± 0.55	
Glucagon	+61 ± 9	−2.63 ± 1.88	
P	<0.005	not significant	

*Numbers in parentheses indicate numbers of perfusions.
Values in μM/gm. with S.E.M. Glucose 10 mM.

DISCUSSION

The chemistry and function of glucagon have been reviewed extensively in recent years;[1,12-14] its acute hyperglycemic effect is known to occur primarily through glycogenolysis. The results of experiments presented here in fasted rats demonstrate that glucagon is able to stimulate hepatic glucose production through gluconeogenesis. In all perfusions glucagon resulted in increased medium glucose and decreased glycogen. The magnitude of the glycogenolytic effect was significant in larger series of experiments but still cannot account for the increase in glucose. The increase in medium glucose and urea in the present studies suggests a relationship between amino acid degradation and glucose synthesis. This effect, in the absence of added amino acid precursors further suggests that glucagon may stimulate mechanisms geared to utilize the liver's own proteins for conversion to glucose. Direct evidence for the use of amino acids as substrate for glucose synthesis is, of course, provided by the demonstration of increased medium glucose specific activity with glucagon in the presence of uniformly labeled l-alanine-C-14. The effect was produced at both trace and substrate quantities of alanine.

Miller first demonstrated in the isolated perfused rat

liver an increase in medium glucose and urea with glucagon.[3] The degree of glucose production was, however, accounted for by extreme glycogenolysis. The increased urea production with glucagon is in contrast to the decrease with insulin, as shown by Mortimore in the perfused rat liver.[15]

Glucagon significantly increased the incorporation of pyruvate-2-C-14 or lactate-1-C-14 into glucose, indicating that the increase in medium glucose in the presence of either substrate resulted from a significant conversion of each into glucose. Since 3-carbon intermediates such as pyruvate and lactate cannot be converted into a 6-carbon hexose without fixation of CO_2[16] glucagon must have stimulated liver CO_2 fixation from bicarbonate in the medium. That this was the case was demonstrated by the increase in medium glucose and increased bicarbonate-C-14 incorporation into glucose when either pyruvate or lactate was perfused in the presence of bicarbonate-C-14.

Noteworthy is the highly significant increase in medium glucose with glucagon in the presence of either fructose or galactose. The magnitude of hyperglycemia with these substrates is about the same order as that seen with either pyruvate or lactate at 10 mM and appears to be higher than that seen with 10 mM alanine. In two out of three perfusions with 10 mM fructose and fructose-C-14, the results of which are not shown here, glucagon resulted in a two- to threefold increase in fructose-C-14 incorporation into glucose and in a two to fivefold increase in glucose formed from fructose. These results suggest that glucagon stimulates conversion of fructose to glucose (and in like manner probably that of galactose to glucose) and is pertinent to the recent observation of Schimassek and Mitzkat[17] who noted that in the perfused rat liver, glucagon resulted in a significant elevation of the glucose-6-phosphate to fructose-1,6 diphosphate ratio.

The increased incorporation of l-alanine-C-14 into glucose with glucagon associated with an increase in urea production suggests stimulation of transaminase activity. Glucagon has indeed been shown to increase the activity of this enzyme.[18] The significant conversion of pyruvate and lactate into glucose also suggests an increase in activity of enzymes in this pathway to glucose. In support of this is the finding of Shrago, Lardy, Nordlie and Foster[19] that glucagon increases the activity of phosphoenolpyruvate carboxykinase, the enzyme that converts oxalacetate to phosphoenolpyruvate, via the oxalacetate having been formed from pyruvate via pyruvate carboxylase. These observations plus the demonstration of increased conversion of fructose or galactose into glucose suggest that glucagon may be capable of stimulating, in parallel, the effective activity of several enzyme systems directed toward gluconeogenesis.

The effects of glucagon in the isolated perfused rat liver, as shown in the present studies, closely resemble those seen with glucocorticoids, but differ in some aspects and probably in the mechanisms involved. Thus, in the perfused rat liver, Miller[3] found that glucagon, but not hydrocortisone, results in increased urea production. Both hormones have a protein catabolic effect,[4] but hydrocortisone causes an elevation of blood alpha amino nitrogen, presumably by increasing peripheral mobilization of amino acids, while glucagon decreases alpha amino nitrogen, presumably by increasing hepatic utilization of amino acids. Hydrocortisone and triamcinolone have been shown to stimulate the conversion of alanine into glucose in rat liver.[20,21] That adrenal steroids are also involved in CO_2 fixation by the liver has been demonstrated by Wagle and Ashmore.[22] Hydrocortisone also increases phosphoenolpyruvate carboxykinase activity in rat liver but does so only after repeated injections, compared to an acute effect of glucagon on the same enzyme.[19] Glucocorticoids possibly also affect the fructose to glucose pathway as suggested by a demonstration of increased fructose-1,6-diphosphatase activity in rabbit liver.[23] These considerations would suggest that although the net effect of both hormones is to increase blood glucose, this is produced with glucagon through both gluconeogenesis and glycogenolysis; on the other hand, with glucocorticoids the hyperglycemia appears to result primarily from gluconeogenesis and decreased peripheral glucose utilization, as has been shown recently by Lecocq, Mebane and Madison.[24] Lastly, it appears from available evidence that the glucocorticoid effect on gluconeogenic enzymes may be adaptive in nature whereas that of glucagon is direct. The rapid action of glucagon, and the numerous substrates which are stimulated by glucagon into gluconeogenesis, suggest that the mode of action of the hormone is on some fundamental process, as if it activated the entire gluconeogenic sequence by some morphological alteration. Morphological changes have, indeed, been demonstrated after glucagon administration.[25]

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