Gluconeogenesis from Fructose in Isolated Rat Liver. Stimulation by Glucagon*

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ABSTRACT: Glucagon was found to stimulate the conversion of fructose (2.0 mM) into glucose in isolated fasted rat liver which had been preperfused with quinolinate (2.4 mM). By inhibiting phosphoenolpyruvate carboxykinase, quinolinate served to minimize, if not prevent, the glucagon stimulation of gluconeogenesis from endogenous precursors requiring that enzyme. During the 10 min following fructose addition, mean glucose formation was 48 μmoles/10 g of liver (std dev 11; N, 7) without glucagon and 101 μmoles/10 g of liver (std dev 14; N, 7) when glucagon was added. Stimulation of glucose release by glucagon could not be accounted for by glycogenolysis as indicated by glycogen measurements. When two livers were perfused with approximately 20 μCi of fructose-*U*-^{14}C for 7 min, one without and one with glucagon, the glucose released in the presence of glucagon contained 45% more radioactivity than the control (4.7×10^6 *vs.* 6.8×10^6). With one pair perfused for 10 min the increment was 67% (6.6×10^6 *vs.* 11×10^6), and with a second pair perfused for 10 min it was 77% (7.9×10^6 *vs.* 14×10^6). Radioactivity measurements of numerous hepatic intermediates failed to disclose the site of action of glucagon. It must be determined if the mechanism by which glucagon stimulates the conversion of fructose into glucose is also responsible for the glucagon stimulation of gluconeogenesis from pyruvate.

Glucagon has been reported to stimulate gluconeogenesis in the isolated rat liver from a variety of precursors including bicarbonate (Garcia *et al.*, 1966), lactate and pyruvate (Schimassek and Mitzkat, 1963; Garcia *et al.*, 1966), and alanine (Garcia *et al.*, 1966; Mallette *et al.*, 1969). On the other hand, it has been reported that glucagon does not stimulate gluconeogenesis from fructose when perfusate fructose concentrations of 10 mM (Ross *et al.*, 1967) or 30 mM (Exton *et al.*, 1970) are used.

In the studies reported in this paper, glucagon stimulated gluconeogenesis from fructose in isolated perfused liver from fasted rats. In most experiments, preperfusion with quinolinate was used to minimize, if not prevent, the flux of carbon from endogenous precursors through the phosphoenolpyruvate carboxykinase reaction (Veneziale *et al.*, 1967). Quinolinate afforded the opportunity to study gluconeogenesis from fructose under conditions in which gluconeogenesis from newly formed lactate and from many endogenous precursors was inhibited by virtue of the inhibition of phosphoenolpyruvate carboxykinase. The initial perfusate fructose concentration was 2.0 mM; by 10 min, the time when most of our observations were made, it had decreased to about 1.3 mM. The latter concentration approximates the average blood fructose concentration resulting from an intravenous infusion of fructose into normal human subjects (Weinstein and Roe, 1952) and the peak value resulting from an oral fructose tolerance test (Stanbury *et al.*, 1966).

Materials and Methods

The livers used in the perfusion experiments were taken from 24-hr-fasted, male, Sprague–Dawley rats weighing 300–400 g. The perfusate (100 ml) consisted of twice-washed bovine erythrocytes suspended (hematocrit value, 30%) in Krebs–Ringer bicarbonate solution (Umbreit *et al.*, 1964) containing 3% (w/v) bovine albumin (Sigma, fraction V powder). The perfusion method, liver sampling techniques, and assays for glucose and various intermediates have been described (Veneziale *et al.*, 1967, 1970; Veneziale, 1971). Fructose was assayed by the method of Klotzsch and Bergmeyer (Bergmeyer, 1965).

Liver glycogen was isolated essentially by the method of Good *et al.* (1933). The precipitated glycogen was hydrolyzed with 4.0 ml of 1.0 N HCl at 100° for 2 hr. The glucose was assayed by the method of Slein (Bergmeyer, 1965). When the entire procedure, including treatment with hot 30% KOH, was applied to 250 μg of standard glycogen samples from rabbit liver (Mann Research Laboratories), the mean recovery was 78% (std dev 1; *N* 7).

Quinolinic acid was purified by multiple recrystallizations from 40% acetic acid followed by complete removal of the solvent. D-Fructose-*U*-^{14}C was obtained from Cal Atomic (Los Angeles, Calif.) and from Amersham–Searle (Des Plaines, Ill.); crystalline glucagon was obtained from Mann Research Laboratories. The glucagon was added to the perfusate as a suspension in 0.15 M NaCl or, in most instances, as a solution in dilute sodium phosphate buffer (pH 10.3).

Paper chromatographic methods alone or combined with high-voltage electrophoresis were utilized to separate the ^{14}C-labeled compounds contained in aqueous, neutralized, perchloric acid extracts of the perfused liver. Five-hundred microliters of extract was quantitatively applied to the origin as follows: the extract was evaporated to approximately 50 μl by a stream of nitrogen and this concentrated extract was applied in 5-μl portions; the transfer was completed with two 30- to 40-μl water washes.

Chromatography system 1 was used for the isolation of

* From the Mayo Clinic and Mayo Foundation, Department of Endocrine Research, Rochester, Minnesota. *Received March 15, 1971.* This paper is II in the series: Hepatic Gluconeogenesis. This investigation was supported in part by grants from the Mayo Foundation and Eli Lilly and Co.

TABLE I: Specific Activity of Selected Compounds by Two Methods.[a]

^{14}C-Labeled Compound	Chromatography System Used for Separation	Sp Act. (dpm/μmole)		% Recov, Method B
		Method A	Method B	
Glutamate	1	200,000	180,000	84
L-(α)-Glycerophosphate	1	16,000	14,000	41[b]
L-(α)-Glycerophosphate	1	15,000	15,000	51[b]
Glucose	1E	78,000	75,000	100
Malate	1E	19,000	16,000	85
Malate	1E	19,000	19,000	106
Glucose	2	17,000	19,000	93
Fructose	2	250,000	260,000	95

[a] Method A: method used in this paper. Method B: after counting labeled compound according to method A, paper was removed from scintillation liquid, air-dried, and eluted with water; one aliquot of eluate was counted in Bray's solution and one was assayed enzymatically. [b] No attempt at complete elution was made.

TABLE II: Rates of Gluconeogenesis During Perfusion with and without Fructose.[a]

Interval (min)	Without Quinolinate		With Quinolinate	
	No Glucagon	Glucagon (5 μg)[b]	No Glucagon	Glucagon (5 μg)[b]
		With Fructose		
0–5	27 ± 12 (5)	37 ± 3 (5)	27 ± 7 (4)	51 ± 14 (4)
0–10	44 ± 14 (5)	90 ± 8 (5)	48 ± 11 (7)	101 ± 14 (7)
0–15	63 ± 20 (6)	116 ± 17 (6)	69 ± 22 (4)	128 ± 14 (5)
		Without Fructose		
0–10	19 ± 4 (3)	23 ± 9 (5)	12 ± 3 (5)	25 ± 8 (6)
0–15	24 ± 4 (3)	29 ± 6 (5)	19 ± 4 (5)	34 ± 10 (6)

[a] Fructose and glucagon were added at 60 min. Intervals are after time of fructose addition to perfusate. Data are given as rate in micromoles formed corrected to 10 g wet wt of liver, means plus or minus standard deviation (*N*). [b] Total perfusate volume, 100 ml.

phosphoenolpyruvate and L-(α)-glycerophosphate, system 2 for isolation of glucose and fructose (Veneziale and Gabrielli, 1969), and system 1E for isolation of 3-phosphoglycerate, glucose 6-phosphate, and fructose 1-phosphate (Veneziale, 1971). The individual species were located by radioautography, and the appropriate areas of the paper were excised and counted with an efficiency of 70% in a Nuclear-Chicago Mark II liquid scintillation counter. Because 3-phosphoglycerate and L-(α)-glycerophosphate migrate together in system 1E, the radioactivity due to the latter, which was determined by system 1, was subtracted to get the radioactivity due to 3-phosphoglycerate. The radioactivity measurement of an intermediate in the extract was corrected to 1 g of tissue and divided by its concentration (micromoles per gram of tissue) to give its specific activity in disintegrations per minute per micromole.

TABLE III: Rates of Gluconeogenesis in Presence of DL-Glycerate.[a]

Interval (min)	No Glucagon	Glucagon (5 μg)
0–15	31 ± 7 (4)	46 ± 14 (5)
0–30	61 ± 22 (4)	76 ± 14 (5)

[a] Quinolinate was added at 30 min; sodium DL-glycerate (perfusate concentration 4.0 mM) was added at 60 min; glucagon was added at 60 min. Data are given as rate in micromoles formed corrected to 10 g wet wt of liver, means plus or minus standard deviation (*N*).

TABLE IV: Utilization of Perfusate Fructose by Isolated Liver.[a]

Fructose at 10 min (μmoles)	No Glucagon	Glucagon (5 μg)
Unused	126 ± 4 (4)	126 ± 6 (4)
Cleared per 10 g of liver	81 ± 10 (4)	87 ± 9 (4)

[a] Quinolinate was added at 30 min; fructose (200 μmoles) and glucagon were added at 60 min; sampling was at 70 min. Data are given as mean values plus or minus standard deviation (*N*).

TABLE V: Effect of Glucagon on Glucose-^{14}C Formation from Fructose-U-^{14}C in Isolated Liver.[a]

Fructose-U-^{14}C Added (dpm/μmole)	Perfusion (min)	Quinolinate	Total Dpm in Perfusate Glucose[b]			
			No Glucagon		Glucagon (5 μg)	
			Value	Expt	Value	Expt
2.1×10^5	10	0	6.3×10^6	19[c]		
2.1×10^5	10	0	5.6×10^6	21[c]	7.5×10^6	22[c]
2.2×10^5	5	+		161		162
2.2×10^5	7	+	4.7×10^6	157[d]	6.8×10^6	158[d]
2.1×10^5	10	+	8.1×10^6	20[c]		
2.1×10^5	10	+	6.6×10^6	155[d]	11×10^6	156[d]
2.4×10^5	10	+	7.9×10^6	163[d]	14×10^6	124[d]
4.5×10^5	10	+	14×10^6	45[c]	23×10^6	46[c]

[a] Quinolinate was added at 30 min; fructose-U-^{14}C and glucagon were added at 60 min. Livers were sampled at 5, 7, or 10 min after addition of fructose-U-^{14}C and extracted with perchloric acid. See Materials and Methods. [b] Corrected to 10 g of liver. [c] Specific activity of extract glucose was corrected to disintegrations per minute per micromole of perfusate glucose and multiplied by total micromoles of perfusate glucose. See Materials and Methods. [d] Glucose contained in aqueous extract of deproteinated perfusate was isolated and its radioactivity measured to determine total disintegrations per minute in perfusate glucose directly. See Materials and Methods.

The ratio of disintegrations per minute per micromole of liver glucose to that of perfusate glucose after 10 min of perfusion with fructose-U-^{14}C under the conditions of our experiments was found to be 1.60 (std dev 0.14; N 7). Using this factor it was possible to correct the specific activity of extract glucose, as experimentally determined, to the specific activity of perfusate glucose. In some experiments the radioactivity in perfusate glucose was then calculated by multiplying its specific activity by the total (micromoles) perfusate glucose. In other experiments the disintegrations per minute in perfusate glucose was determined directly by isolating the glucose contained in the aqueous extract of deproteinated perfusate (Somogyi, 1945) and measuring its radioactivity. To accomplish this, 0.2 ml of perfusate was added to 1.8 ml of H_2O followed by the addition of 1.0 ml each of $Ba(OH)_2$ and $ZnSO_4$; 0.5 ml of the supernatant was applied to chromatograms and the glucose was isolated by means of chromatography system 2 as described above.

The method used for determining the specific activity of a compound depends on a high percentage recovery of that compound after its chromatographic separation. By using ^{14}C-labeled standards, it was determined that the mean recoveries of radioactivity in system 1 were: malate, 87% (std dev 3; N 6); aspartate, 91% (std dev 5; N 6); phosphoenolpyruvate, 75% (std dev 2; N 3); and 3-phosphoglycerate, 96% (std dev 5; N 6). The recoveries were less than 100% because, in each case, labeled contaminants were present as demonstrated by inspection of the radioautograms. The recoveries of L-(α)-glycerophosphate, glucose 6-phosphate, and fructose 1-phosphate are believed also to be high because, when stained with Hanes spray (Hanes and Isherwood, 1949), multiple standard chromatograms of these species yielded in each case only the expected single discrete blue spot. Table I gives additional evidence of the accuracy of our method for determining specific activity.

Results

Rates of gluconeogenesis during perfusion with fructose are given in Table II. In most cases the rate was approximately doubled as a result of the addition of glucagon. In contrast, the rate of gluconeogenesis in the presence of added glycerate, a stable product of fructose metabolism (Heinz *et al.*, 1968), was not influenced by glucagon to this extent (Table III).

Hepatic glycogen content resulting from perfusion with

TABLE VI: Hepatic Metabolites and Adenine Nucleotides During Perfusion with Fructose.[a]

Compound	Concentration (μmoles/g wet wt)	
	No Glucagon	Glucagon (5 μg)
Lactate	0.21 ± 0.03 (3)	0.11 ± 0.02 (5)
Pyruvate	0.14 ± 0.03 (4)	0.034 ± 0.010 (5)
Phosphoenolpyruvate	0.14 ± 0.03 (5)	0.20 ± 0.04 (5)
2-Phosphoglycerate	0.032 ± 0.017 (5)	0.045 ± 0.024 (5)
3-Phosphoglycerate	0.31 ± 0.06 (5)	0.41 ± 0.06 (5)
Malate	0.21 ± 0.02 (3)	0.24 ± 0.03 (5)
Aspartate	0.76 ± 0.13 (4)	0.85 ± 0.04 (5)
L-(α)-Glycerophosphate	0.096 ± 0.014 (3)	0.086 ± 0.010 (5)
Fructose 6-phosphate	0.021 ± 0.002 (4)	0.020 ± 0.005 (5)
Glucose 6-phosphate	0.049 ± 0.005 (4)	0.072 ± 0.007 (5)
ATP	2.54 ± 0.22 (4)	2.81 ± 0.31 (5)
ADP	0.81 ± 0.11 (5)	0.80 ± 0.12 (5)
AMP	0.17 ± 0.05 (4)	0.18 ± 0.04 (5)

[a] Quinolinate was added at 30 min; fructose and glucagon were added at 60 min. Livers were sampled at 75 min and extracted with perchloric acid. See Materials and Methods for details. Results are shown as means plus or minus standard deviation (N).

TABLE VII: Incorporation of ^{14}C From Fructose-*U*-^{14}C Into Glucogenic Metabolites in Isolated Rat Liver.[a]

Perfusate addn[b]	Expt	Units	Fructose-1-P (Dpm/g)	Lactate	Phosphoenol-pyruvate	3-Phospho-glycerate	L-(α)-Glycero-phosphate	Glucose-6-P
Q, Fru-*U*-^{14}C	161	Dpm/μmole	44,000	NV[c]	38,000	37,000	33,000	130,000
		μmole/g		0.060	0.12	0.29	0.077	0.048
Q, Fru-*U*-^{14}C, G	162	Dpm/μmole	42,000	NV	32,000	38,000	30,000	140,000
		μmole/g		0.095	0.26	0.56	0.084	0.045
Q, Fru-*U*-^{14}C	157	Dpm/μmole	37,000	9700	28,000	49,000	33,000	
		μmole/g		0.24	0.20	0.40	0.069	0.042
Q, Fru-*U*-^{14}C, G	158	Dpm/μmole	39,000	5200	25,000	35,000	35,000	160,000
		μmole/g		0.12	0.21	0.45	0.069	0.050
Q, Fru-*U*-^{14}C	155	Dpm/μmole	31,000	7000	42,000	37,000	49,000	180,000
		μmole/g		0.20	0.20	0.43	0.058	0.037
Q, Fru-*U*-^{14}C,G	156	Dpm/μmole	31,000	4900	36,000	35,000	23,000	90,000
		μmole/g		0.21	0.23	0.47	0.12	0.097
Q, Fru-*U*-^{14}C	163	Dpm/μmole	42,000	3,600	32,000	43,000	8100	130,000
		μmole/g		1.08	0.17	0.38	0.49	0.041
Q, Fru-*U*-^{14}C, G	164	Dpm/μmole	56,000	6800	34,000	48,000	45,000	210,000
		μmole/g		0.21	0.66	1.36	0.10	0.048

[a] See Table V for description of experiments. In expt 163, liver was found to be in a highly reduced state as evidenced by a lactate:pyruvate ratio of 19.6. The liver of expt 164 had unusually high concentrations of phosphoenolpyruvate and 3-phosphoglycerate for no apparent reasons. All weights refer to wet weight of liver. [b] Q = quinolinate; G = glucagon. [c] NV = not visible by radioautography.

fructose was determined in livers which were preperfused with quinolinate. At 60 min, just before fructose was added, the mean glycogen content was 11 μmoles (glucose)/10 g wet wt (std dev 5; *N* 7). At 70 min, 10 min after fructose addition, it was 18 μmoles/10 g (std dev 7; *N* 6). Therefore, the glucagon-induced increment in rate of glucose formation in the presence of fructose (Table II) cannot be accounted for by glycogenolysis.

The rate of utilization of fructose by the isolated rat liver is given in Table IV. After 10-min perfusion, approximately 40% of the added fructose had been extracted from the perfusate, both in the absence and in the presence of glucagon.

Glucose-^{14}C formation from fructose-*U*-^{14}C was studied primarily in the presence of quinolinate (Table V). The use of this inhibitor afforded the opportunity to observe the effects of glucagon on fructose-*U*-^{14}C metabolism at a time when flux through the phosphoenolpyruvate carboxykinase reaction was minimized, if not completely inhibited. Glucagon did stimulate gluconeogenesis from fructose.

The concentrations of hepatic metabolites and of the adenine nucleotides during perfusion with quinolinate and fructose in the absence and in the presence of glucagon are given in Table VI. Two effects of glucagon are evident. There was a decrease in the concentrations of lactate and pyruvate, greater in the case of pyruvate. The ratio of glucose 6-phosphate to fructose 6-phosphate increased, which might have been due partly to an increased flux from the latter to the former and partly to stimulation of glycogenolysis.

The incorporation of isotope from fructose-*U*-^{14}C into some glucogenic metabolites is given in Table VII. Glucagon did not consistently increase the label in the fructose 1-phosphate pool over that found in the controls. From the specific activity of fructose-*U*-^{14}C perfused and the measured radioactivity due to the fructose 1-phosphate in 1 g wet wt of the liver, it can be deduced that the concentration of this metabolite was approximately 0.2 μmole/g wet wt of liver. Therefore, no accumulation of it occurred, in contrast to the experience of others (Woods *et al.*, 1970) under different conditions. The limited labeling of 3-phosphoglycerate relative to that of glucose 6-phosphate indicates that 3-phosphoglycerate and, probably, glycerate are not major metabolites in the conversion of fructose into glucose. The limited labeling of L-(α)-glycerophosphate relative to glucose 6-phosphate suggests that there is considerable dilution of cytoplasmic glycerophosphate by unlabeled mitochondrial glycerophosphate (Klingenberg, 1970). An alternative explanation—limited conversion of dihydroxyacetone phosphate into glycerophosphate—seems unlikely in view of the equilibrium constant for that reaction as given by Bücher and Hohorst (Bergmeyer, 1965).

Discussion

The failure of others to observe a glucagon-induced stimulation of gluconeogenesis from fructose has been used to support suggestions ". . .that the stage between pyruvate and oxalacetate is the site of action of this glucagon effect" on pyruvate metabolism (Ross *et al.*, 1967) and that a ". . . physiologically meaningful stimulation of reactions [by glucagon] between triose-P and glucose seems unlikely" (Exton *et al.*, 1970). In contrast, Garcia *et al.* (1966) reported that in the presence of fructose (initial perfusate concentration, 10 mM), in two of three perfusions, glucagon resulted in a two- to threefold increase in fructose-^{14}C conversion into glucose, although it was not stated how perfusate glucose-^{14}C could be counted as the osazone presumably in the presence of unused perfusate fructose-^{14}C. Furthermore, in those long-term experiments (2 hr), without an inhibitor of lactate gluconeo-

genesis, it is possible that glucagon was stimulating glucose formation from lactate that had been produced by fructolysis. Our data (Tables II and V) demonstrate conclusively that glucagon can stimulate the conversion of fructose into glucose and can do it at a concentration of substrate more nearly that which is encountered physiologically. This observation makes it reasonable to speculate that at least some of the stimulation of gluconeogensis from pyruvate by the hormone is a result of the same mechanism which causes stimulation of gluconeogensis from fructose.

It seems unlikely that the glucagon effect on fructose utilization was to increase the supply of fatty acids for oxidation. Fructose and glucose exist at the same level of oxidation; therefore, the biosynthesis of glucose from fructose cannot require a net quantity of reducing equivalents. The mechanism could involve the hexose phosphates as indicated by the increase observed in the ratios of glucose 6-phosphate to fructose 6-phosphate (Table VI) and of glucose 6-phosphate to fructose diphosphate (Schimassek and Mitzkat, 1963), although this must be considered as only speculation at this time.

The data of Table VII do not disclose the site(s) of stimulation by glucagon. More is being learned about the influence of the hormone on fructose metabolism in isolated liver by using specifically labeled fructose.

The discovery that pancreatic glucagon can be inappropriately secreted (Unger *et al.*, 1970) makes it of potential medical importance that the site(s) and mechanism(s) of action of the hormone on hepatic gluconeogenesis be fully understood. Perhaps augmented gluconeogenesis secondary to inappropriate secretion of glucagon is a primary cause of hyperglycemia in some patients.

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