STUDIES ON THE KETOGENIC EFFECT OF GLUCAGON IN INTACT RAT LIVER*

J. R. Williamson, B. Herczeg, H. Coles, and R. Danish

The Johnson Research Foundation, University of Pennsylvania
Philadelphia, Pennsylvania

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The most striking effect of glucagon administered to a well-fed animal is a prompt rise of blood sugar due to the activation of liver phosphorylase. Glucagon has also been reported to have proteolytic, gluconeogenic, lipolytic and anti-lipogenic effects, but until very recently the mechanism for these diabetogenic effects of glucagon was not clear (Foa, 1964). The present study was prompted by a report that ketogenesis was stimulated in liver slices following glucagon administration to rats in vivo, and that liver homogenates contain a glucagon sensitive lipase (Bewsher et al., 1966).

Methods. Male Holtzman rats weighing between 220 and 280 g were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body wt), and the femoral vein was cannulated. Crystalline glucagon (lot no. 258-234B-167-1, courtesy of Eli Lilly and Co.) was dissolved in Krebs bicarbonate Ringer and injected intravenously at the rate of 50 µg/min for the first minute followed by 5 µg/min for total periods of 5, 30, and 60 minutes. Control rats were treated similarly with infusions of Krebs bicarbonate Ringer. The livers were rapidly frozen in situ with tongs pre-cooled in liquid N_2. The powdered tissue was extracted once with 4 vol. 8°/o $HClO_4$ in 40°/o ethanol, and once with 3 vol. 6°/o $HClO_4$. The combined supernatants were neutralized to pH 5.5 with 3 M K_2CO_3 containing 0.5 M triethanolamine. The residue after perchloric acid extraction was washed once with 0.6°/o perchloric acid, once with water, and suspended in 3 vol. 5 mM mercaptoethanol. Long chain fatty acyl CoA derivatives in the residue were hydrolyzed to free CoA by the addition of 1 N KOH containing 5 mM mercaptoethanol to bring the pH to 10.5-11.0. The mixture was allowed to stand for 20 minutes at 30° with occasional mixing, and neutralized to pH 5.0 with 6°/o $HClO_4$. A clear supernatant was obtained after centrifugation for 10 min at 30,000 g.

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Coenzyme A was measured fluorometrically by a minor modification of the α-ketoglutarate oxidase method described by Garland et al. (1965). Acetyl CoA was assayed similarly using citrate synthase (Wieland and Weiss, 1963). With the high sensitivity of the fluorometric method, the correction factor described by Buckel and Eggerer (1965) in the acetyl CoA assay was found to be unnecessary. Recovery of CoA and acetyl CoA from the tissue extracts was quantitative. Other intermediates were measured using standard enzyme procedures (Bergmeyer, 1963).

Results. Table 1 shows that glucagon increased tissue levels of acetyl CoA and fatty acyl CoA, and decreased those of free CoA in livers from fed rats.

TABLE 1

EFFECTS OF GLUCAGON ON FED RAT LIVER IN VIVO

Glucagon dose = 50 μg for 1 min followed by 5 μg/min continuous infusion.

Treatment	Time (min)	CoA	Acetyl CoA	Acyl CoA
		mμmoles/g dry wt		
Control	5	230 ± 23	131 ± 14	73 ± 6
Glucagon	5	162 ± 5	191 ± 9	97 ± 14
Change	5	-68*	+60*	+24
Control	30	220 ± 9	124 ± 9	72 ± 9
Glucagon	30	148 ± 18	219 ± 7	97 ± 6
Change	30	-72*	+95*	+25*
Control	60	163 ± 9	147 ± 5	82 ± 9
Glucagon	60	197 ± 13	143 ± 17	109 ± 3
Change	60	+34	-4	+27*

*p = or less than 0.05; 4 rats per group.

Glucagon increased ketone levels in livers of fed rats: there being a 2-fold increase of acetoacetate and β-hydroxybutyrate after 5 min, and a 3-fold increase after 30 min (Table 2). Plasma FFA also increased, from 355 ± 80 to 621 ± 59 mμmoles/ml after 30 min of glucagon infusion. Oxalacetate levels tended to decrease with glucagon, while citrate levels increased almost 2-fold 30 min after the start of glucagon infusion (Table 2). As observed with acetyl CoA, the glucagon effects on the levels of ketones, oxalacetate and citrate seen at earlier times were no longer apparent after 60 min.

TABLE 2

KETOGENIC EFFECTS OF GLUCAGON ON FED RAT LIVER IN VIVO

Glucagon dose = 50 μg for 1 min followed by 5 μg/min continuous infusion.

Treatment	Time (min)	Acetoacetate	β-Hydroxybutyrate	Oxalacetate	Citrate
		mμmoles/g dry wt			
Control	5	346 ± 35	387 ± 23	21.6 ± 2.5	749 ± 18
Glucagon	5	670 ± 55	1045 ± 249	15.5 ± 1.5	778 ± 56
Change	5	+324*	+658*	-6.1*	+29
Control	30	403 ± 47	417 ± 50	——	784 ± 145
Glucagon	30	1110 ± 168	1286 ± 78	——	1319 ± 69
Change	30	+707*	+869*	——	+535*
Control	60	766 ± 82	900 ± 128	22.2 ± 0.9	1407 ± 56
Glucagon	60	813 ± 167	962 ± 227	19.1 ± 1.3	1223 ± 88
Change	60	+47	+62	-3.1	-184

*p = or less than 0.05; 4 rats per group.

TABLE 3

EFFECTS OF STARVATION ON RAT LIVER IN VIVO

Condition	CoA	Acetyl-CoA	Acyl-CoA	Oxal-acetate	Citrate	Total ketones
	mμmoles/g dry wt					
Fed	202 ± 7	134 ± 5	76 ± 4	21.9 ± 1.2	840 ± 89	1105 ± 147
Unfed 24-30 hours	186 ± 9	400 ± 15	122 ± 8	16.5 ± 2.0	1657 ± 136	6700 ± 440
Change	-16	+266*	+46*	-5.4*	+817*	+5595*

*p = or less than 0.05; 8 to 16 rats per group.

Glucagon (infused for times up to 60 min) had no effect on the levels of CoA, acetyl CoA and acyl CoA in livers from rats unfed for 24-30 hours. Likewise, glucagon had no effect on plasma FFA levels in unfed rats, the mean control values being 806 ± 72 mμmoles/ml. There was, however, an increase in the level of total ketones in unfed rats with glucagon; control values being 6.27 ± 0.47 and 7.13 ± 0.75 μmoles/g dry wt after 5 and 60 minutes, respectively, while with glucagon values of 7.67 ± 0.52, 9.01 ± 1.12 and 10.61 ± 0.50 μmoles/g dry wt were obtained after 5, 30, and 60 minutes, respectively.

Table 3 shows a comparison of intermediates found in the fed and unfed

groups of rats. The levels of acetyl CoA, acyl CoA, ketones and citrate all increased in the unfed group, while oxalacetate levels decreased. The free CoA levels, on the other hand, failed to decrease in proportion to the increase of bound CoA, so that there was a considerable increase of total CoA with starvation.

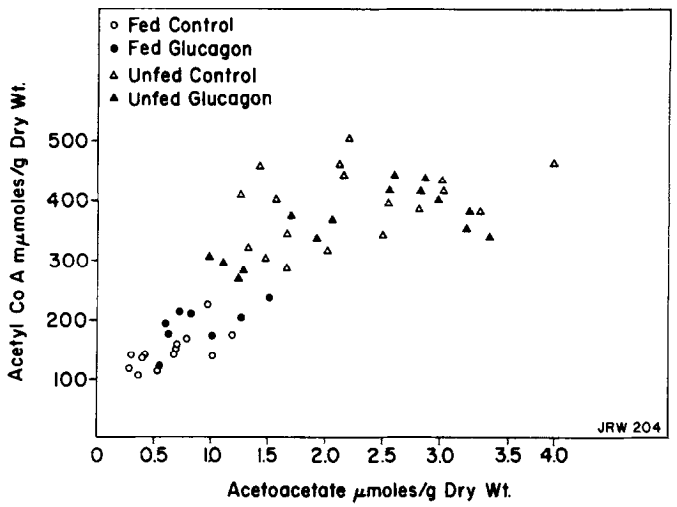

Fig. 1. Correlation of acetyl CoA and acetoacetate levels in rat liver.

In Fig. 1 the acetyl CoA levels are plotted against the acetoacetate levels in the various groups of animals. Although the scatter is considerable, it is apparent that acetoacetate increased in proportion to acetyl CoA up to acetyl CoA levels of about 400 mμmoles/g dry wt, and thereafter increased with little further rise of acetyl CoA.

Discussion. The present results demonstrate that glucagon stimulates the formation of ketone bodies in intact livers from fed rats. The increased ketogenesis was associated with elevated tissue levels of acetyl CoA and long chain fatty acyl CoA. The enhanced ketogenesis of starvation was also associated with increases of acetyl CoA and fatty acyl CoA, as previously observed by Bortz and Lynen (1963) and Tubbs and Garland (1964). Similarly, the livers of diabetic cortisol treated rats have been shown to have elevated acetyl CoA levels (Wieland and Weiss, 1963). The relationship between acetyl CoA and acetoacetate appeared to be linear when the acetyl CoA levels were low, but at higher levels saturation occurred, so that a rise of acetoacetate was not accompanied by a further rise of acetyl CoA. A somewhat similar relationship has recently been reported by Garland et al. (1965) in studies with isolated

rat liver mitochondria oxidizing palmitylcarnitine. These findings may mean that at low acetyl CoA levels, acetoacetate is formed primarily by the deacylation of acetoacetyl CoA, while at higher acetyl CoA, the alternative pathway involving the condensation of acetyl CoA with acetoacetyl CoA to form β-hydroxy-β-methylglutaryl-CoA, proposed by Lynen et al. (1958) is also operative.

Glucagon is known to stimulate glycerol and free fatty acid release from adipose tissue (Vaughan and Steinberg, 1963). Adipose tissue has been shown to contain a lipase, sensitive to stimulation by epinephrine, glucagon and ACTH (Vaughan et al., 1964). These hormones are thought to activate the enzyme through the release of cyclic 3,5 AMP, since this nucleotide may replace the hormone as an activating agent (Rizack, 1964). Cyclic 3,5 AMP levels have recently been shown to be elevated in adipose tissue treated with epinephrine or glucagon (Butcher et al., 1966). Similarly, a hormone-sensitive lipase has been described in liver, which also responds to cyclic 3,5 AMP (Bewsher and Ashmore, 1966). It seems probable, therefore, that the ketogenic action of glucagon is brought about partly by an increase in the concentration of fatty acids in the blood and partly by a direct lipolytic activity of the hormone in the liver. The present findings and those of Struck et al. (1965) who observed a stimulation of ketogenesis by glucagon in the isolated perfused rat liver support this possibility. Both glucagon and free fatty acids have been shown to stimulate gluconeogenesis from lactate and alanine in the isolated rat liver (Struck et al., 1965; Garcia et al., 1966; Williamson et al, 1966), hence the gluconeogenic effect of glucagon may be secondary to enhanced lipolysis and proteolysis.

Insulin has a well-documented anti-lipolytic effect in adipose tissue (Mahler et al., 1964), and recent work by Butcher et al. (1966) indicates that this effect is produced by decreasing the tissue level of cyclic 3,5 AMP. The transient effect of glucagon on ketogenesis and acetyl CoA levels in the intact rat liver reported here, and the contradictory reports of glucagon effects on lipolysis and ketogenesis in the literature (Foa, 1964) may be explained by insulin release elicited by glucagon, either directly, or secondarily to the elevated blood sugar levels.

Palmityl-CoA has been shown to inhibit citrate synthase activity in vitro (Wieland et al., 1964; Tubbs and Garland, 1964; Srere, 1965). The present study shows that both with glucagon and after starvation, when fatty acyl CoA levels are elevated, citrate levels also increase, despite a concurrent fall of oxalacetate. The recent report by Fritz (1966) that long chain acyl carnitine derivatives relieve palmityl CoA inhibition of citrate synthase, is of interest in this connection. The concentration of acyl carnitine derivatives

in liver is not known, but Pearson and Tubbs (1964) have presented preliminary data showing that acetyl carnitine levels increase in rat liver with starvation.

Summary. Glucagon administered by intravenous infusion to rats in vivo has been shown to stimulate ketogenesis and increase levels of acetyl CoA and fatty acyl CoA in the liver. This effect was observed in fed rats, where it was prompt and transient, but not in rats unfed for 24 hours. These results substantiate that glucagon has a lipolytic effect in liver. The ketogenesis induced either by glucagon or starvation was associated with elevated levels of citrate and decreased levels of oxalacetate. A relationship between acetyl CoA and acetoacetate levels in the liver is described.

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