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Further Studies Concerning Glucagon-Induced Hypocholesterolemia[1] (38762)

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We reported (1) that glucagon has a prompt hypocholesterolemic effect in normal rats fed a stock diet, and that it prevents the hypercholesterolemia observed in this species after the administration of a high cholesterol diet or after thyroidectomy or hypophysectomy. In accord with these experimental observations, Davignon (2) recently reported that the plasma cholesterol of some hypercholesterolemic patients was reduced by the chronic administration of glucagon.

Our initial study did not reveal the mechanism responsible for this hypocholesterolemic property of glucagon. Therefore we began investigating the influence of glucagon upon various aspects of cholesterol metabolism. The results of these studies, described below, suggest that glucagon acts in several ways to reduce the serum cholesterol concentration of the rat.

I. *Methods. A. Effect of glucagon on the incorporation of* ^{3}*H-acetate into liver cholesterol.* 1. *Acute Effect.* Twelve rats were fasted overnight, bled for serum cholesterol assay and then six of them were injected subcutaneously with 0.1 mg of glucagon (Eli Lilly). Six hours later, all 12 were bled again and then injected intravenously with 0.5 mCi Na acetate ^{3}H.[2] One hour following the last injection, the rats were killed and their livers perfused free of blood with 0.9% NaCl solution. The concentration of cholesterol in liver and serum was determined and the specific radioactivity of digitonin-precipitable sterols in the liver was determined according to previously described methods (3).

2. *Chronic effect.* After obtaining control blood samples from 10 rats, five of them received two daily subcutaneous injections of 1.0 mg of glucagon. All rats were offered, and consumed completely, 30 ml of a low-cholesterol liquid diet (4) per day. Seven days later, after an overnight fast, after a second bleeding, and 1 hr after the final injection of glucagon, all rats were given Na acetate ^{3}H as described above. The rats were killed 1 hr after the injection of the labeled acetate and their livers were perfused and removed. Cholesterol and radioactivity in the serum and livers were assayed as described previously (3).

B. *Effect of glucagon on the rate of intestinal absorption of cholesterol.* After obtaining blood samples from 36 normal rats, 18 of them were injected twice daily with 0.1 mg of glucagon. All rats were offered, and consumed completely, 30 ml per day of the low-cholesterol liquid diet (4). Seven days later, after an overnight fast, after a second bleeding, and 1 hr after the last injection of glucagon, all rats were given 0.085 mCi of ^{3}H-cholesterol (1.9×10^8 dpm)[3] dissolved in 1 ml of cottonseed oil, by stomach tube.

All rats were bled 4 and 8 hr after the administration of the tracer and were killed immediately thereafter. Their livers were perfused and removed and their gastro-intestinal tracts as well as all feces excreted during the 8-hr period of study were obtained. The amount of cholesterol absorbed during the 8-hr period was calculated from the fraction of tracer cholesterol remaining in the gastro-intestinal tract and feces. The amount of labeled cholesterol present in the livers also was determined as previously described (3). The two blood samples obtained prior to the ingestion of tracer cholesterol were assayed for serum cholesterol concentration and the two samples obtained after cholesterol feeding were assayed for radioactivity and cholesterol concentration.

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[2] New England Nuclear Company, 100 mCi per m*M*.

[3] Amersham Searle, Arlington Heights, IL, 500 mCi/m*M*.

C. *Effect of glucagon on the rate of disappearance of cholesterol from plasma.* The effect of glucagon upon the rate of disappearance of cholesterol from plasma was studied using two types of labeled cholesterol: (1) lipoprotein cholesterol-^{3}H of endogenous origin and (2) chylomicron cholesterol-^{3}H.

In a first series of experiments, after collecting a control blood sample for serum cholesterol analysis, 13 of 27 rats were injected subcutaneously with 0.1 mg of glucagon twice daily for 7 days. The other rats were injected with 0.9% NaCl solution. Both experimental and control rats were given a diet free of cholesterol[4] to minimize possible differences in their intestinal absorption of cholesterol.

Seven days after beginning the glucagon injections and approximately 1 hr after the last glucagon injection had been given and after cannulation of the bile duct under ether anesthesia, the rats were injected intravenously with 2 ml of serum containing lipoprotein cholesterol-1α,2α ^{3}H (each dose contained 9.1×10^5 dpm of ^{3}H-cholesterol). The bile secreted during the first hour after cannulation was discarded in order to decrease the pool of preformed bile acids. The bile secreted during the next 5 hr was collected. All rats were bled immediately after the injection of the ^{3}H-cholesterol and again 6 hr later. Then they were killed and their livers were perfused with 0.9% NaCl solution and removed. Cholesterol was determined quantitatively in each serum, bile and liver sample and the specific radioactivity was calculated from measurements of the radioactivity of cholesterol separated by partition between solvents (5). The bile acid content of the bile samples was determined quantitatively by the method of Talalay (6) as modified by Admirand and Small (7). The radioactivity of bile acids was determined after separation from cholesterol according to the method of Folch *et al.* (5).

The labeled lipoprotein cholesterol preparation was produced by injecting 0.5 mCi of cholesterol 1α,2-^{3}H dissolved in 1,2-propanediol into the peritoneum of three normal rats, and bleeding the animals 48 hr later. The distribution of radioactivity in the serum lipids was determined by thin layer chromatography employing the methods of Folch *et al.* (5), Chedid *et al.* (8), and Parker and Peterson (9). Twenty five percent of it was found in the cholesterol fraction and 75% in the cholesterol esters of the pooled serum.

To study the possible effect of glucagon upon the clearance of chylomicron cholesterol from plasma, 25 rats were bled to obtain samples for cholesterol analysis, after which 12 rats were injected subcutaneously with 0.1 mg of glucagon twice daily for 7 days. The other rats were injected with 0.9% NaCl solution. These rats also received the cholesterol-free diet described above.

Seven days after beginning glucagon injections, all 25 rats were fasted overnight, anesthetized with ether and an indwelling polyethylene catheter was inserted into the right iliofemoral vein. The rats then were placed in restraining cages. Six hours later, after recovering from anesthesia, all rats were injected through the catheter with 1 ml of pooled rat intestinal lymph containing chylomicrons labeled with ^{3}H-cholesterol, according to a previously described technique (10). This pooled lymph contained 56 mg of cholesterol/100 ml and 3.3×10^8 dpm/ml. Radioactivity was measured in blood samples obtained 4, 8, 12, 16 and 32 min following such injections. This was done by pipetting 0.05 ml of whole blood into 10 ml of a scintillation fluid composed of toluene and ethanol (7:3, v/v), containing Omnifluor (3.5%, w/v, New England Nuclear Co., Boston, MA). The radioactivity was measured in a Packard Model 3003 scintillation counter. Quench corrections were calculated after addition of known quantities of toluene-^{3}H. The initial blood sample was assayed also for its plasma cholesterol concentration.

Immediately after the last bleeding, the rats were killed, their livers were perfused

[4] Simonsen Laboratory Guinea-pig Chow. Ingredients: 1. pulverized heavy oats; 2. whole wheat; 3. alfalfa leaf meal, U.S. No. 1, 17% protein, sun cured or dehydrated; 4. dehulled soy bean oil meal, containing not less than 50% protein; 5. sodium chloride, iodized; 6. limestone; 7. Nopco Vitamin Premix. Analysis: Moisture $\leq$10%; fibre $\leq$13.5%; fat, 2.5–4.0%; ash $\leq$9%; protein $\geq$19.0%; calcium, 1.53%; phosphorus, 0.27%; iodine, 0.0033%; salt, 0.75–1.0%.

with 0.9% NaCl and assayed for radioactivity and for total cholesterol concentration (3).

II. *Results. A. Effects of glucagon on the incorporation of* ^{3}*H-acetate into hepatic cholesterol.* 1. *Acute effects.* Within 6 hr of a single injection of glucagon, the plasma cholesterol concentration of six rats declined significantly ($P < 0.001$) from 56 ± 2.1 SEM to 33 ± 3.2 SEM mg/100 ml; while that of the six control rats remained essentially the same: 58 and 56 mg/100 ml. The incorporation of ^{3}H-acetate into hepatic cholesterol was virtually the same in the two groups. Thus, one hour after the injection of ^{3}H-acetate, the average dpm/mg of hepatic cholesterol of the glucagon-treated rats was $9.54 \times 10^3 \pm 1.94 \times 10^3$ SEM; that of the control rats was $10.2 \times 10^3 \pm 1.5 \times 10^3$ SEM.

2. *Chronic effects.* The average plasma cholesterol concentration of five rats given glucagon for 7 days declined significantly ($P < 0.001$) from 54 ± 1.9 SEM to 29 ± 3.0 SEM mg/100 ml. The initial average plasma cholesterol (50 mg/100 ml) of the five control rats remained essentially the same (56 mg/100 ml). Despite the marked fall in the plasma cholesterol content of the glucagon-treated rats, their average incorporation of ^{3}H acetate into hepatic cholesterol did not differ significantly from that of the untreated rats. Thus, 1 hr after the injection of the labeled acetate, the average dpm/mg of hepatic cholesterol in the glucagon treated rats was $2.69 \times 10^3 \pm 0.63 \times 10^3$ SEM, and in the control rats was $2.0 \times 10^3 \pm 0.41 \times 10^3$ SEM.

B. *Effect of glucagon on the rate of intestinal absorption of cholesterol.* As Table I illustrates, two daily injections of 0.1 mg of glucagon for 7 days led to a modest, but statistically significant ($P < 0.05$) lost in body weight and to a significant ($P < 0.005$) fall in plasma cholesterol. Table I also indicates that the chronic administration of glucagon markedly reduced the rate of intestinal absorption of cholesterol. Thus, the glucagon-treated rats absorbed approximately 20% and the control rats, approximately 35% of the administered labeled cholesterol in 8 hr. This inhibition of absorption probably was the reason why the average amount of labeled cholesterol present in the plasma of glucagon-treated rats, 4 and 8 hr after ingestion, was significantly less than that found in the plasma of the untreated rats. Similarly, the hepatic content of labeled cholesterol in the glucagon-treated rats was significantly smaller than that found in the control rats.

C. *Effect of glucagon on the rate of disappearance of cholesterol from plasma or blood.* 1. *Lipoprotein cholesterol.* Endogenous produced lipoprotein cholesterol disappeared more rapidly from the blood of glucagon-treated rats than from the blood of the untreated rats (Table II). Thus, 6 hr after intravenous injection, the radioactivity present in the plasma of the experimental rats was less than half of that present in the plasma of the control rats. In conformity with these results, the liver content of labeled cholesterol, 6 hr after its injection, was significantly greater in the livers of the experimental rats than in the livers of the control rats. However the total amounts of labeled and unlabeled bile acids excreted by the glucagon-treated rats were not significantly different from those excreted by the control rats. Similarly the biliary excretion of labeled and unlabeled cholesterol was essentially the same in both groups of rats.

2. *Chylomicron cholesterol.* Unlike the endogenously produced lipoprotein cholesterol, the labeled cholesterol present in lymph chylomicrons did not disappear (Table III) any faster from the blood of glucagon-treated rats than from the blood of the control rats. As might be expected from these results, the hepatic accumulation of labeled cholesterol, 32 min after its injection, was essentially the same in the two groups of animals.

Discussion. In our initial publication (1) we reported that chronic administration of glucagon lowered the plasma cholesterol concentration of both hypercholesterolemic and normocholesterolemic rats; but we had no data concerning the mechanism(s) which might bring about this effect.

In the present study, several possible mechnisms have been detected. The first one is a decrease in the rate of cholesterol absorption. Thus 8 hr after the oral administration of labeled cholesterol, glucagon-treated rats were found to have significantly more un-

TABLE I. THE EFFECT OF GLUCAGON ON RATE OF INTESTINAL ABSORPTION OF CHOLESTEROL.

No. of rats	Average weight (g)		Plasma cholesterol (mg/100 ml)		Intestinal absorption of ^{3}H cholest. (8 hr)	DPM × 10^{-6}/ml Plasma		Liver				
	Before glucagon	7 Days after glucagon	Before glucagon	7 Days after glucagon	(per cent)	4 hr after feeding ^{3}H-cholesterol	8 hr after feeding ^{3}H-cholesterol	Avg. wt.	Cholesterol (mg/total liver)	dpm × 10^{-5} mg Chol.	dpm × 10^{-6} total liver cholesterol	% of adm. ^{3}H chol. in liver
A. Rats given repeated injections of glucagon												
18	295	269[a]	65	29[b]	19.8[b]	0.31[b]	1.18[b]	9.1	25.3	2.8[b]	6.9	4.1[b]
Range:	(277–313)	(243–288)	(58–80)	(16–39)	(2–55)	(0.21–0.53)	(0.34–2.6)	(7.9–10.8)	(18.0–34.4)	(1.1–4.8)	(2.6–12.4)	(1.7–8.2)
SE Mean:	±3.2	±3.6	±1.6	±1.6	±2.9	±0.03	±0.14	±0.2	±1.0	±0.2	±0.5	±0.4
B. Control rats												
18	290	283	67	74	35	0.85	2.51	10.3	26.2	4.3	11.3	7.0
Range:	(270–316)	(262–370)	(55–84)	(58–100)	(13–70)	(0.38–1.40)	(0.92–4.51)	(8.4–12.4)	(19.2–33.2)	(2.2–7.1)	(5.2–21.8)	(2.5–14.3)
SE Mean:	±3.6	±4.6	±2.2	±4.2	±3.4	±0.09	±0.24	±0.3	±1.1	±0.4	±1.2	±0.9

[a] Average value significantly ($P < 0.05$) different from that of control value.
[b] Average value significantly ($P < 0.005$) different from that of control value.

TABLE II. EFFECT OF GLUCAGON ON RATE OF DISAPPEARANCE FROM PLASMA OF LIPOPROTEIN ^{3}H-CHOLESTEROL AND ITS CONVERSION INTO BILE ACIDS.

No. of rats	Average weight (g)		Plasma Cholesterol (mg/100 ml)		dpm × 10^{-4}/ml plasma (after injection of ^{3}H-cholesterol)		Liver				Bile					
	Before glucagon	7 days after glucagon	Before glucagon	7 days after glucagon	Immediate	6 hours	Average weight (g)	Cholesterol mg/g	dpm × 10^{-5} total liver cholesterol	% of dose	Average volume (5 hr)	Bile Acids mg/ml	Bile Acids mg/total volume	dpm × 10^{-4} Total bile acids	Cholesterol mg/ml	dpm × 10^{-4} total bile cholesterol
A. Rats given repeated injections of glucagon																
13	281	273	59	33[a]	6.6	0.90[a]	10.3	2.5	3.43[a]	32.5[a]	4.2	11.06	45.2	3.65	0.35	1.54
Range:	(258–308)	(242–311)	(50–68)	(26–49)	(4.6–8.3)	(0.1–1.9)	(7.10–12.1)	(2.2–2.8)	(2.94–3.94)	(23.5–39.2)	(2.6–6.4)	(5.5–16.4)	(20.7–72.2)	(0.8–5.6)	(0.18–0.44)	(1.49–1.61)
SE Mean:	±5.4	±6.6	±3.6	±2.0	±0.4	±0.1	±0.4	±0.1	±0.1	±1.2	±0.3	±1.5	±5.5	±0.5	±0.1	±0.1
B. Control rats																
14	276	291	61	65	7.0	2.2	10.7	2.3	2.18	20.3	4.7	11.4	51.0	2.62	0.37	1.58
Range:	(250–308)	(255–326)	(48–80)	(48–83)	(0.52–0.87)	(0.15–2.5)	(9.38–11.78)	(2.1–2.9)	(1.74–2.89)	(14.6–26.2)	(4.0–6.0)	(7.67–18.9)	(30.7–79.8)	(0.9–4.35)	(0.20–0.47)	(1.44–1.62)
SE Mean:	±5.9	±7.01	±2.3	±3.7	±0.4	±0.1	±0.4	±0.1	±0.1	±0.86	±0.2	±3.7	±3.7	±0.4	±0.1	±0.1

[a] Significantly ($P < 0.001$) different from comparable control value.

TABLE III. EFFECT OF GLUCAGON ON RATE OF DISAPPEARANCE FROM BLOOD OF CHYLOMICRON CHOLESTEROL.

No. of rats	Average weight (g)	Average plasma cholesterol (mg/100 ml)		After injection of intestinal lymph containing ^{3}H-cholesterol-bearing chylomicrons								
				Plasma (dpm × 10^{-2}/0.05 ml plasma)					Liver			
											dpm × 10^{-4}	
		Before glucagon	7 Days after glucagon	(min) 4	8	12	16	32	Average weight (g)	Cholesterol (mg/Gm)	per mg cholesterol	per total liver
A. Rats given repeated injections of glucagon												
12	312	70	40[a]	11.7	9.4	7.8	6.0	2.8	11.4	2.1	6.3	149
Range:	(271–333)	(56–85)	(29–77)	(8.4–15.5)	(5.4–15.1)	(3.9–11.0)	(2.3–10.7)	(1.2–5.6)	(8.8–14.1)	(1.73–2.8)	(3.7–9.6)	(85–213)
SE Mean:	±5.5	±2.5	±3.8	±0.9	±0.9	±0.9	±0.8	±0.4	±0.7	±0.1	±0.5	±15.6
B. Control rats												
13	320	71	58	11.3	9.4	8.2	6.9	3.4	11.6	2.0	6.9	148
Range:	(303–343)	(47–95)	(46–72)	(6.3–16.2)	(5.1–15.4)	(3.3–13.8)	(2.8–12.4)	(1.3–7.0)	(7.6–15.1)	(1.3–2.7)	(3.8–10.4)	(78–214)
SE Mean:	±3.6	±4.1	±3.3	±1.0	±1.0	±1.0	±0.9	±0.6	±0.6	±0.1	±0.7	±14.6

[a] Significantly different from control value ($P < 0.001$).

absorbed cholesterol in their gastrointestinal tract and feces than did the control rats. It is probable that the lower concentration of cholesterol in the plasma of treated rats was due to lessened intestinal absorption rather than to more efficient hepatic extraction of chylomicrons and subsequent catabolism, because when labeled chylomicron cholesterol was injected intravenously, the labeled substance appeared in the liver of treated rats at the same rate as it did in that of the control animals.

Inhibition of cholesterol absorption cannot be the only mechanism by which glucagon lowers plasma cholesterol, because plasma cholesterol was already significantly reduced, six hours following a single injection of glucagon into previously fasted rats. Also, intravenously injected endogenous lipoprotein cholesterol (soluble in plasma, in contrast to chylomicron cholesterol) disappeared more rapidly from the plasma of the glucagon-treated rat than from the plasma of the control rats. This more rapid disappearance could have been due to a greater ability of the glucagon-treated rat to remove (and possibly catabolize) soluble plasma cholesterol. Or, as suggested by Eaton (11), it could have been due to a reduced capacity of the liver of the glucagon-treated rat to synthesize and discharge cholesterol-bearing lipoproteins. Since we did not observe any acceleration in the conversion of cholesterol into bile acids by the livers of our glucagon-treated rats, we are inclined to believe that the second of these mechanisms was at play, that is, a reduced rate of re-entry of the labeled cholesterol from the liver into the plasma. This would account for the fact that, 6 hr following injection of labeled lipoprotein cholesterol, there was less labeled cholesterol in the plasma and more in the liver of the glucagon-tested rats.

Glucagon did not alter either the rate of disappearance of intravenously administered chylomicron cholesterol from the plasma or its rate of hepatic accumulation. This apparent difference in the behavior of injected chylomicron cholesterol and of lipoprotein cholesterol might be due to the fact that the rates of disappearance and hepatic accumulation of labeled cholesterol was measured for only 32 min after the injection of the chylomicron cholesterol (because of the short half-life of plasma chylomicrons) whereas, after the injection of the lipoprotein cholesterol, these rates were measured for a 6 hr period. On the other hand, the difference could be due to diverse transport pathways of chylomicron and lipoprotein cholesterol.

Summary. Some of the possible mechanisms responsible for the hypocholesterolemic effect of glucagon were investigated. Glucagon was found to inhibit the intestinal absorption of cholesterol. In addition, it was found to either hasten the rate of egress of lipoprotein cholesterol from the blood into the liver or to retard the rate of re-entry of cholesterol from the liver into the blood. The data do not distinguish between these two possibilities, which indeed may occur simultaneously.

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