Negative Feedback in Sterol Biosynthesis and Lipoprotein Release by the Perfused Rat Liver[1] (37324)

LEE A. BRICKER, PATRICIA L. KOZLOVSKIS, AND MICHAEL G. GOODMAN
(Introduced by Gerald S. Levey)
Department of Medicine, University of Miami School of Medicine, Biscayne Annex, Miami, Florida 33152

The cholesterol negative-feedback mechanism, whereby dietary cholesterol inhibits *de novo* hepatic sterol formation (1–6), has been well described. Although this system has been studied by a variety of *in vitro* techniques, the relationship between the total amount of hepatic sterol synthesized to that released into the circulation under conditions of variable cholesterol intake has not been previously defined. In the present experiments *in situ* perfusion of the isolated rat liver was employed to examine this relationship.

Materials and Methods. Sprague–Dawley rats were fed diets consisting of high cholesterol (5%) in ground rat chow (with 10% oleic acid to aid absorption of cholesterol) or of ground rat chow with 10% oleic acid, containing negligible amounts of cholesterol. The cholesterol used in these diets, purchased from Nutritional Biochemicals, Inc., was recrystallized 3× from ethanol to enhance purity; to this was added 1α,2α-^{3}H-cholesterol (Ambersham-Searle, Inc.) to produce a final specific activity of 3670 (±5%) dpm/μmole cholesterol. Diets were maintained for 3 to 4 wk. At the time of perfusion, the rat was given an anesthetic dose of sodium pentobarbital intraperitoneally, and surgically prepared. The perfusion apparatus was of variable pressure and constant flow type (Vanderbilt University apparatus shop). The perfusion technique has been previously described (7). The perfusion medium (100 ml) consisted of outdated, washed human red cells in a medium containing 4 g bovine Fraction V albumin (Armour) and 60 mg glucose, in Krebs bicarbonate buffer adjusted to pH 7.40 by the addition of 1.25 *N* NaOH. The final hematocrit was approximately 20%. The perfusate (rate 7 ml/min) was oxygenated with humidified oxygen (95%, with 5% CO_2). The entire system was maintained at 38°. The 0.5 ml samples of perfusate were drawn from the sampling portal with 1.0 ml syringes at 0, 5, 10, 15, 25, 35, 45, 55, 65, 75, 90, 120, 150, and 180 min after commencing infusion of radioactive sodium acetate-2-^{14}C into the venous side of the circuit.

Isotope infusion into liver perfusion system. Sodium acetate-2-^{14}C, of original specific activity 2.0 μCi/μmole, was mixed with a nonradioactive sodium acetate carrier to yield a final specific activity of 0.30 μCi/μmole. This lower-specific activity isotope, dissolved in Krebs bicarbonate buffer, was infused from a 5 ml syringe with a Braun-Melsungen pump directly into the venous-return side of the apparatus, into the drum-oxygenator. This was done as follows: A priming dose was administered in a steady 1 min pulse of 0.5 ml containing 6.25 μCi ^{14}C-acetate. After this first minute, the infusion pump speed was reduced to 0.02 ml (0.25 μCi/min) for the remaining 179 min. A final total of 51.0 μCi was thereby infused throughout the 3-hr duration of the experiment, in 169.0 μmoles of the sodium acetate and in a total infusion volume of 4.08 ml.

At the end of the perfusion, the liver was rapidly cut free of the carcass, weighed, and frozen. Portions of the liver (1.0 g) were later minced, extracted three times in hot 2:1 chloroform:methanol, and the combined ex-

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tracts made up to a specific volume (100 ml). An aliquot was saponified, extracted with petroleum ether and assessed for total cholesterol as described previously (8).

Methanol solutions of the resulting sterol digitonides were divided into two aliquots. One of these was measured colorimetrically, and the other was placed in a scintillation medium consisting of POPOP, 0.15%; PPO, 0.3%; ethyl acetate, 6.5%; and toluene, 93.5% (w/v) and counted in a Packard Model 3390 liquid scintillation spectrometer equipped with an "absolute activity analyzer." Appropriate quench curves were obtained to permit simultaneous assessment of tritium and ^{14}C.

To each of the 0.5 ml perfusate samples (unseparated, containing red cells) was added 0.25 mg nonradioactive cholesterol carrier to make possible subsequent digitonin precipitation. Lyophilized total bile collections were handled similarly. These samples were saponified in 1.5 ml of ethanol:10 *N* KOH, 2:1 (v/v), and extracted as above with petroleum ether. Digitonin precipitates were made, washed, and counted *in toto*. The cpm value in each 0.5 ml sample was corrected for total volume in the system at its specific point in time, based on measurement of the final total perfusate volume and assumed linear rate of loss. This allowed an assessment of newly synthesized digitonin-precipitable sterol then present in the total circulation and its accumulation with time.

Results. Figure 1 illustrates the individual rates of accumulation of newly synthesized digitonin-precipitable sterol in the perfusate circulation. Approximately 45–55 min elapsed before radioactive sterol could be detected. Data for total acetate-2-^{14}C incorporation into sterols, both stored and released by 3 hr, are shown in Table I. In each experiment, newly synthesized sterol within the liver was considerably in excess of that released to the perfusate during the 3-hr time period, usually by a factor of eightfold or more. Despite the "feedback-induced" differences in ^{14}C-acetate incorporation into sterols, both stored and released to the circulation, the tendency of the liver to retain most of the ^{14}C-sterol is unaffected by diet, as indicated by the

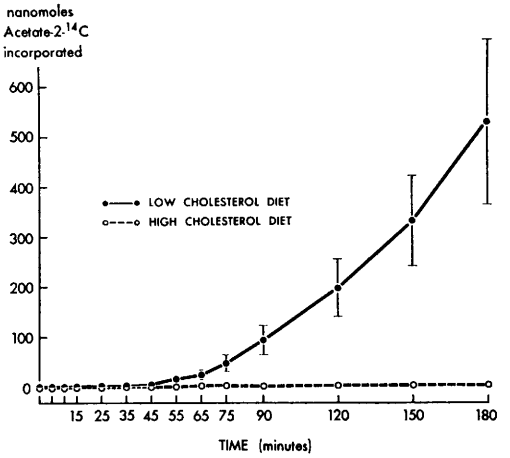

FIG. 1. Appearance of *de novo* digitonin-precipitable sterol (from incorporated acetate-2-^{14}C) in the total circulation of the perfused liver preparation. Data are from 6 rats on low cholesterol diet and 7 on high cholesterol diet. Vertical bars represent ± 1 standard error of the mean.

lower part of Table I. Only a tiny fraction of the *de novo* sterol appeared in the bile at 3 hr, although a higher fraction of the total was found in bile from high cholesterol-fed donors than from low. The reasons for this apparent relative insensitivity of biliary sterologenesis to feedback suppression in the perfused liver are unclear.

Table II illustrates further the property of the perfused liver to release a relatively small proportion of its sterol into the circulation. In this instance tritium in the digitonin precipitates was analyzed. Since the only source of tritium in these studies was dietary cholesterol, the data illustrate the fate of this dietary cholesterol stored in liver at the beginning of the perfusion. At the end of 3 hr, less than 2% of the stored hepatic cholesterol had been mobilized into the circulation.

Discussion. This study demonstrates both the relationship of dietary cholesterol to hepatic new sterol synthesis in the perfused liver and the relationship between newly synthesized sterols stored in liver to those released to the perfusion medium in circulating lipoproteins. The perfusion system has proved a satisfactory method of demonstrating hepatic sterol release (9, 10), and has provided a new technique for studying the

TABLE I. Effects of Low and High Cholesterol Diets on Synthesis and Release of *de Novo* Sterols by the Perfused Liver.

Cholesterol in diet (%)	Rat no.	Liver wt (g)	Total sterol content (mg/g liver)	Acetate-2-^{14}C incorporated into digitonin-precipitable sterol (nmoles/3 hr)			Distribution of newly synthesized digitonin-precipitable sterol in 3 hr		
				Storage in whole liver	Released to Circulation	Released to Bile	% Released to Circulation	% Released to Bile	% Retained in liver
0	1	13.2	3.0	5432	409.0	2.80	7.0	0.05	93
	2	16.0	3.1	3920	127.2	0.20	3.1	0.004	97
	3	12.8	2.1	6144	244.0	1.21	3.8	0.02	96
	4	10.0	2.3	9200	1106.0	3.10	10.7	0.03	89
	5	10.5	3.2	11,865	945.0	11.60	7.4	0.09	93
	6	7.1	3.6	6319	348.0	4.08	5.5	0.06	94
Mean		11.6	2.9	7147	529.9	3.80	6.3	0.04	94
± SE		1.3	0.2	1176	162.9	1.70	1.1	0.01	1
5	1	14.4	—	119.5	1.78	0.097	1.5	0.007	98
	2	14.6	19.6	299.3	14.41	0.184	4.8	0.06	95
	3	22.0	26.3	66.0	9.40	0.030	12.5	0.04	87
	4	15.7	28.4	69.1	7.30	—	8.9	—	91
	5	15.8	26.1	189.6	4.85	1.040	2.5	0.53	97
	6	14.8	25.3	122.8	1.48	0.635	1.2	0.51	98
	7	15.5	23.1	169.0	0	0	0	0	100
Mean		16.4	24.8	147.9	5.60	0.332	4.5	0.19	95
± SE		1.1	1.3	30.6	1.94	0.170	1.7	0.10	2

cholesterol feedback phenomenon. Earlier work (11) has shown that consequences of this phenomenon are demonstrable in circulating blood *in vivo*, but the timing and extent to which appearance of new sterol in blood reflects total sterologenesis has not been previously estimated. These data demonstrate that during a 3-hr perfusion, the great majority of new sterol is retained in liver. Since the liver is presumably at equilibrium regarding storage, synthesis, and release of sterol at the commencement of perfusion, it seems unlikely that this partitioning of new sterol predominantly into an intrahepatic site is due to a perfusion-induced artifact. Roheim, Miller and Eder (12) in their studies on plasma lipoprotein formation in the perfused liver of the cholesterol-fed rat showed that the protein in the perfusion medium in all likelihood subserves the func-

TABLE II. Distribution of Dietary Cholesterol Initially Stored in Rat Liver, After 3 hr of *in Situ* Perfusion.

Rat no.	Liver wt (g)	^{3}H-Cholesterol/liver (μmoles)	^{3}H-Cholesterol in total circulation (μmoles)	Total ^{3}H biliary cholesterol (μmoles)	% of Total ^{3}H-cholesterol in circulation
1	14.0	2885	19.6	0.020	0.68
2	14.8	2220	13.8	0.040	0.62
3	14.8	1935	9.0	0.004	0.47
4	15.5	2802	11.2	0.044	0.40
5	19.9	1892	79.8	0.057	4.20
Mean	15.8	2347	26.7	0.033	1.27
± SE	1.1	210	13.4	0.009	0.73

tion of a lipoprotein precursor. Had whole heparinized rat blood or $d > 0.21$ proteins plus red cells been used in place of the albumin–erythrocyte system we employed, a somewhat greater cholesterol release would thereby have been expected. In fact, *in vivo* studies, accomplished by infusing acetate-2-^{14}C intravenously into intact rats, have shown this to be the case (Bricker, L. A., unpublished data). In this instance, of course, native blood circulated to the liver, and still over 85% of the total new ^{14}C-sterol in liver plus blood was found in the substance of the liver.

The measurements presented here reveal only the extent to which administered acetate-2-^{14}C is incorporated into digitonin-precipitable sterol, and not, of course, actual rates of synthesis. Despite the large amount of nonradioactive acetate used as carrier, the data may nonetheless underestimate the total two-carbon flux throgh the sterologenic pathway. While it is conceivable that the two diets may result in differential rates of dilution of labeled acetate by endogenous, unlabeled acetyl CoA, thereby giving rise to misleading indications of rates of sterol synthesis, earlier work directed precisely at this point makes such a consideration unlikely. Using an *in vitro* system, Siperstein and Guest (13) showed that acetate-2-^{14}C could be incorporated into ketone bodies as well as into fatty acids and CO_2 at rates independent of prior dietary cholesterol or of concurrent sterologenesis. Since, under conditions of differing cholesterol intake no significant differences in specific radioactivity of ketone bodies generated from radioacetate were found, it would appear unlikely that the initial acetyl CoA pool sizes could have been affected by the diet. Our conclusion that rates of incorporation of radioacetate into digitonin-precipitable sterol parallels actual rates of sterol synthesis would thereby appear tenable.

Summary. A high cholesterol diet (5%) given to rats over a 3- to 4-wk period markedly suppressed incorporation of acetate-2-^{14}C in the perfused liver into both stored hepatic cholesterol and into sterol released into the perfusion medium. Perfused livers from low cholesterol-fed rats incorporated radioacetate into sterols at rates at least 50 times those observed in livers of high cholesterol-fed rats. In all animals, during the 3-hr perfusion period, only a small fraction of newly synthesized sterol was released from liver, the remainder being stored within it. Newly synthesized sterol was not detectable in the circulating perfusate until 45–55 min had elapsed. The data demonstrate the cholesterol feedback phenomenon in the perfusion fluid of the perfused rat liver, the quantitative relationship between stored and released new sterols, and the minimal appearance time required for newly synthesized lipoprotein to appear in the circulation of this perfused system.

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