

Origin of milk cholesterol in the rat: dietary versus endogenous sources

R. CLARENBURG and I. L. CHAIKOFF

Department of Physiology, University of California, Berkeley, California

ABSTRACT Slices of mammary gland from lactating rats, incubated with acetate-1-¹⁴C or mevalonate-2-¹⁴C, synthesized cholesterol-¹⁴C.

Within 2 min of an intravenous injection of 1 ml of a suspension of chylomicrons containing cholesterol-4-¹⁴C, mammary glands of lactating rats removed as much as 29% of the labeled cholesterol; those of 2-day-postlactating rats removed none.

Rats were fed a diet containing 0.05% cholesterol-4-¹⁴C from 7 days prepartum to 20 days postpartum. At isotopic equilibrium, the relative specific activities of milk and dietary cholesterol indicated a dietary origin for 11% of the milk cholesterol. The extent to which endogenous sources—liver and mammary gland—contributed cholesterol to milk proved entirely dependent on whether dietary cholesterol, in the form of chyle lipoproteins, was first processed by liver or taken up directly by mammary gland. Lack of information regarding the extent to which chyle cholesterol is removed from blood under physiological conditions by mammary gland and other tissues precludes precise assessment of the endogenous contributions to milk cholesterol and, moreover, casts doubt on the quantitative interpretability of cholesterol-¹⁴C-feeding experiments reported in the literature.

KEY WORDS lactation · rat · mammary gland · cholesterol · biosynthesis · uptake · chylomicron · dietary · cholesterol-4-¹⁴C · liver · isotopic equilibrium · difficulties · interpretation

NUMEROUS STUDIES with isotopes have left no doubt that the lactating mammary gland has an exceptionally high capacity for synthesizing fatty acids (1). It has also been shown that chylomicron triglycerides are taken up by the lactating mammary gland and transported into milk (2–4). However, information on the origin of cholesterol in rat milk is limited to a recent study (5) in

which cholesterol-¹⁴C was fed to rats and, when isotopic equilibrium had been established, the origin of milk cholesterol was calculated from the specific activities of cholesterol in milk, plasma, and diet.

The present report deals with (a) *de novo* synthesis of cholesterol by liver and mammary gland of the lactating rat, (b) direct uptake by mammary gland and liver of the lactating rat of cholesterol-4-¹⁴C from an intravenously injected, chylomicron preparation labeled with cholesterol-4-¹⁴C, and (c) the equilibration of labeled cholesterol among plasma, liver, mammary gland, and milk of rats fed a diet containing 0.05 % cholesterol-4-¹⁴C from 7 days prepartum to 20 days postpartum. An objection to an earlier interpretation (5, 6) of cholesterol-¹⁴C-feeding experiments is raised, and an alternative interpretation is proposed. It is concluded that about 11% of the milk cholesterol is of dietary origin and that the extent to which the endogenous sources—liver and mammary gland—contribute cholesterol to milk cannot be determined from a cholesterol-¹⁴C-feeding experiment so long as the proportions of dietary cholesterol taken up in the form of chyle lipoproteins by liver and mammary gland under physiological conditions are not known.

I. STEROL SYNTHESIS FROM LABELED PRECURSORS BY SLICES PREPARED FROM MAMMARY GLANDS AND LIVERS OF LACTATING RATS

Experimental Methods

Lactating Long-Evans rats, weighing 240–380 g and nursing at least six pups, were fed a stock diet (Diablo Labration) for the first 10 days of lactation. For the next 5 days they were fed either this diet or diet A shown in Table 1. Postlactating rats (that had suckled at least six pups for 21 days and were killed 48 hr after their litters had been weaned) and virgin rats received the stock diet.

Abbreviations: DPS, digitonin-precipitable sterols; HCC, higher counting companions (of cholesterol).

TABLE I COMPOSITION OF SPECIAL DIETS

Constituent	Diet A	Diet B
	%	%
Ground whole wheat	67.5	67.5
Casein (technical, vitamin-free)	15.0	15.0
Skim milk powder	7.5	7.5
NaCl*	0.75	0.75
CaCO ₃	1.5	1.5
Melted fat (hydrogenated vegetable oil)	6.75	7.5
Fish oil†	1.0	
Aqueous vitamin solution‡		0.20
Labeled cholesterol		0.05

* Sufficient KI solution was added to the NaCl to furnish 0.9 micrograms I⁻ per gram of diet.

† Contained 750 IU of vitamin A and 300 IU of vitamin D per gram.

‡ Parke, Davis & Co., Detroit, Mich. Each 0.6 ml of this solution contains 5000 IU of vitamin A and 1000 IU of vitamin D.

lipids were extracted from the incubated tissues by the method of Hotta, Hill, and Chaikoff (11). Digitonin-precipitable sterols were isolated from the total lipid extracts and purified according to Sperry and Webb (12). The digitonides were dissolved in known volumes of anhydrous methanol. The sterol contents of the methanol solutions were determined by the FeCl₃ color reaction (13); their ¹⁴C contents were measured in a Packard liquid scintillation spectrometer by mixing 1 ml of each solution with 15 ml of toluene containing 45 mg of 2,5-diphenyloxazole and 1.5 mg of 1,4-bis[2-(5-phenyloxazolyl)]-benzene. Quenching due to the methanol was taken into account. In some instances cholesterol was purified by bromination (14) and its specific activity determined.

TABLE 2 INCORPORATION OF PRECURSOR ¹⁴C INTO CO₂, TOTAL LIPIDS, AND DIGITONIN-PRECIPIABLE

The amount of tissue slices recorded in column 5 was incubated for 3 hr at 37° with 5 ml of Krebs-Henseleit bicarbonate buffer, pH 7.4. Results are expressed as means

Expt. No.	No. of Rats	State of Rat*	Diet† Fed	Amount of Tissue Incubated	¹⁴ C-Labeled Compound Added‡		Unlabeled Glucose Added
					Kind	Amount	
1	3	Lactating	Stock	1.0	Acetate	0.17	0
					Mevalonate	0.71	0
2	3	Lactating	Diet A	1.5	Acetate	0.19	0
					Mevalonate	0.77	0
3	6	Lactating	Stock	0.75	Acetate	50.1	0
				0.75	Acetate	50.1	50
	6	Postlactating	Stock	0.75	Acetate	50.1	0
				0.75	Acetate	50.1	50
	6	Virgin	Stock	0.75	Acetate	50.1	0

* Lactating; killed on the 16th day of lactation while nursing at least six pups. Postlactating; killed 48 hr after removal of the litter, which had been nursed for 21 days.

† Stock diet was Diablo Labration. Composition of diet A is given in Table 1.

‡ The specific activity of the acetate-1-¹⁴C used was about 10 μc/μmole; that of the DL-mevalonate-2-¹⁴C, 1.2 μc/μmole. Half the amounts of DL-mevalonate ¹⁴C added are listed here because only one of the optical isomers of DL-mevalonate is metabolized.

The rats were stunned by a blow on the head and bled from the neck area. Their livers and mammary glands were rapidly removed and immersed in an ice-cold, Krebs-Henseleit bicarbonate buffer (7) of pH 7.4. Tissue slices prepared with the aid of a McIlwain-Buddle tissue chopper (8) were washed repeatedly with the buffer solution and blotted with filter paper. Weighed portions of the slices were incubated with shaking for 3 hr at 37° in flasks (9) containing the buffer solution and the sodium salt of either acetate-1-¹⁴C or DL-mevalonate-2-¹⁴C. ¹⁴CO₂ was collected and measured as described by Entenman, Lerner, Chaikoff, and Dauben (10). Total

Results

The results of three experiments are presented in Table 2. In Experiment 1, about 0.2 μmole of labeled acetate was incubated with 1.0 g of slices obtained from livers and mammary glands of the rats fed the stock diet. In both tissues about 30% of the added ¹⁴C was recovered as CO₂, but the recoveries of ¹⁴C as digitonin-precipitable sterols (DPS) of liver and mammary gland were considerably different, being 6 and 1% of the added acetate-¹⁴C, respectively. Even greater was the difference between the two organs in the formation of ¹⁴C-DPS from mevalonate-2-¹⁴C.

In Experiment 2, we measured the formation of ^{14}C -DPS in livers and mammary glands of rats fed the same diet as that used in the cholesterol- ^{14}C equilibration experiment (Section III). A 1.5 g portion of slices was incubated with approximately the same concentration of labeled acetate and mevalonate as that used in Experiment 1. The results were similar to those of Experiment 1.

In liver, almost all of the ^{14}C -labeled DPS formed from acetate-1- ^{14}C was precipitated by bromination (only 2.5% was found in HCC¹), but in the mammary gland as much as 40% of the ^{14}C -DPS was present as HCC (Table 2, Experiment 2). Higher percentages of HCC were found in the ^{14}C -DPS formed from mevalonate-2- ^{14}C in both tissues—13% in liver and 73% in lactating mam-

concentrations of unlabeled cholesterol precursors may be present in the former tissue.

In Experiments 1 and 2, small amounts (less than 1 μmole) of labeled precursors were used. In the third experiment, however, 50 μmoles of acetate were added (to 0.75 g of tissue slices) in order to maximize uptake of labeled acetate by tissue slices and to approach saturation of their sterol-forming systems. A comparison of the results obtained with the tissues of lactating rats in Experiment 3 with those of Experiments 1 and 2 shows that: (a) similar percentages (about 30%) of the added acetate were converted to CO_2 by the liver, but much less (about 6.5%) by the mammary gland. This finding is in accord with the conclusion of Abraham and Chaikoff (17) that the oxidation of acetyl units via the Krebs

STEROLS (DPS) BY MAMMARY GLAND AND LIVER SLICES OF LACTATING AND CONTROL RATS

and the additions listed. The gas phase was 95% O_2 and 5% CO_2 . Duplicate portions of liver and mammary gland slices were incubated, and their standard errors.

Liver			Mammary Gland			
% of ^{14}C Recovered per Flask as:		% HCC§ in ^{14}C -DPS	% of ^{14}C Recovered per Flask as:			% HCC§ in ^{14}C -DPS
CO_2	DPS		CO_2	Lipids	DPS	
30.1 ± 0.74	5.93 ± 0.56	—	27.6 ± 0.72	—	0.95 ± 0.07	—
6.17 ± 0.24	38.7 ± 1.42	—	0.39 ± 0.02	—	2.61 ± 0.12	—
34.1 ± 0.54	5.39 ± 0.67	2.5 ± 0.2	31.9 ± 0.67	—	0.89 ± 0.11	40.0 ± 5.3
6.07 ± 0.19	37.5 ± 2.91	12.8 ± 2.6	0.32 ± 0.04	—	2.25 ± 0.52	73.0 ± 4.9
30.6 ± 2.78	0.34 ± 0.07	—	6.60 ± 1.21	0.69 ± 0.19	0.027 ± 0.004	—
—	—	—	6.31 ± 1.71	34.0 ± 1.78	0.056 ± 0.010	—
27.3 ± 2.16	0.09 ± 0.02	—	2.12 ± 0.16	0.14 ± 0.02	0.002 ± 0.0004	—
—	—	—	1.83 ± 0.13	0.67 ± 0.06	0.003 ± 0.0010	—
27.7 ± 3.26	0.27 ± 0.05	—	—	—	—	—

§ ^{14}C in the noncholesterol fraction of the DPS, calculated as follows:

$$\% \text{ HCC} = \frac{\text{Specific activity of DPS minus specific activity of purified cholesterol}}{\text{Specific activity of DPS}} \times 100$$

mary gland. Several findings² have led to the view that HCC contain sterol intermediates that are involved in the conversion of acetate to cholesterol (15, 16). Therefore, our finding of higher percentages of HCC in the ^{14}C -DPS formed in the mammary gland than in the ^{14}C -DPS formed in the liver suggests that HCC turnover is slower in the mammary gland than in liver; larger

cycle in slices of lactating rat mammary gland is saturated at a low acetate level; and (b) the difference between liver and mammary gland in ^{14}C -DPS formation was more pronounced when the large amount of labeled acetate was used. Even under these conditions, however, the incorporation of acetate- ^{14}C into DPS by the lactating mammary gland was of greater significance than reported earlier by Hirsch, Baruch, and Chaikoff (18).

The addition of 50 μmoles of unlabeled glucose to the incubation medium resulted in a 50-fold stimulation in synthesis of total ^{14}C -lipids from acetate-1- ^{14}C in the lactating gland. This confirms earlier findings (17–19).

¹ The term higher counting companions of cholesterol (HCC) denotes digitonin-precipitable ^{14}C -sterols which are not precipitated by bromination (14).

² Clarenburg, R., and I. L. Chaikoff. Papers submitted.

TABLE 3 REMOVAL OF CHOLESTEROL FROM INTRAVENOUSLY INJECTED CHOLESTEROL-4-¹⁴C-LABELED

The experimental conditions are described in the text. The chylomicron preparation used in Experiment 1 contained 84,200 cpm of ¹⁴C per ml, of which tained 120,000 cpm/ml, 56.9% of which was in the form of esters. The ¹⁴C recoveries are expressed as percentages of injected ¹⁴C; the specific activities

Expt. No.	Rat No.	Body Weight	State of Rat*	Time Killed after Injection	Mammary Gland					
					Digitonin-Precipitable Sterols			Specific Activity		
					¹⁴ C			Total	Free	Ester
					Per Whole † Organ	Per g Organ	% Ester			
1	1	290	Lactating	11	10.8	0.93	15.5	0.47	0.40	>7.2
	2	365	"	11	5.3	0.36	16.7	0.21	0.17	>3.1
2	1	255	Postlactating	12	1.1	0.11	44.4	0.04	0.03	0.16
	2	270	"	12	1.2	0.11	46.8	0.05	0.03	0.17
	3	230	Lactating	2.5	12.1	1.31	63.7	0.83	0.32	>41
	4	262	"	2.5	27.3	2.60	60.5	1.60	0.64	>78
	5	260	"	12	16.7	1.60	56.0	1.03	0.46	>45
	6	306	"	12	31.9	2.60	61.2	1.06	0.41	>79
3	1	280	Lactating	2	9.1	0.81	54.4	—	—	—
	2	330	"	2	28.8	2.18	52.9	—	—	—
	3	300	"	2	20.1	1.68	50.3	—	—	—
	4	340	"	11	9.9	0.73	26.8	—	—	—
	5	310	"	11	8.3	0.67	29.6	—	—	—
	6	352	"	11	9.5	0.68	23.9	—	—	—

* See Table 2.

† Total mammary gland weight was estimated as 4% of the body weight (see text); total plasma volume was based on a total blood volume of 6.7% of the rat weight (41) and a hematocrit of 0.4 as found in some of the (lactating) rats.

However, the incorporation of acetate-¹⁴C into DPS in the lactating gland was no more than doubled by the addition of glucose.

The incorporation of acetate-¹⁴C into DPS by the mammary glands of rats 48 hr after the litters had been weaned was severely depressed compared to that observed with the lactating glands. The production of ¹⁴CO₂ and ¹⁴C-fatty acids was also decreased in the postlactating glands (see reference 17), and the addition of 50 μmoles of unlabeled glucose to the incubation medium—though causing a 5-fold stimulation in incorporation of acetate ¹⁴C into total lipids—hardly affected the production of ¹⁴C-DPS.

The synthesis of ¹⁴C-DPS per gram of liver was similar for lactating and virgin rats (Table 2). In lactating rats, however, the liver is enlarged in proportion to the number of pups in the litter (see below) so that sterol synthesis per whole liver in the lactating rat may well be above normal. A depressed incorporation of acetate-¹⁴C into DPS by livers of the postlactating rats was found.

In Experiments 1 and 2, about 0.002 μmole of the added labeled acetate was incorporated into DPS by the lactating gland, and about 0.012 μmole by liver. In Experiment 3, in the presence of glucose, about 0.03 μmole of the added labeled acetate was incorporated into DPS by the lactating gland, and about 0.17 μmole by the liver. Thus, the extent of biosynthesis of ¹⁴C-sterols from the labeled acetate by the lactating mam-

mary gland was about one-sixth that by liver³ under our experimental conditions. The results of these isotope experiments do not necessarily reflect the relative capacities of liver and mammary gland to synthesize cholesterol. Differences in uptake of the labeled acetate, acetate pool sizes, and dilution of labeled intermediate compounds by unlabeled intermediates formed by pathways other than those for cholesterol synthesis may explain the differences observed between liver and mammary gland slices in their extent of acetate-¹⁴C incorporation into DPS. Our experiments do demonstrate, however, that the lactating mammary gland is capable of synthesizing cholesterol from acetate.

II. UPTAKE OF CHOLESTEROL FROM INTRAVENOUSLY INJECTED CHYLOMICRONS BY LIVERS AND MAMMARY GLANDS OF LACTATING RATS

Experimental Methods

After its thoracic duct had been cannulated (21), a donor rat received by stomach tube 50 μc (about 4 μmoles) of cholesterol-4-¹⁴C dissolved in 0.5 ml of olive oil. Chyle was collected, defibrinated, and layered under 9 volumes of saline (d₂₀ = 1.006), and the mixture was

³ Addition of glucose to the liver slices was omitted, since extensive formation of glucose has been reported (20) in liver slices prepared from adequately fed animals and incubated under conditions similar to those of the present experiment.

CHYLOMICRONS BY LIVERS AND MAMMARY GLANDS OF LACTATING AND POSTLACTATING RATS

54.7% was in the cholesterol ester fraction; that of Experiment 2 contained 267,000 cpm/ml, 58.2% of which was esterified; that of Experiment 3 contains percentages of injected ¹⁴C per mg sterols.

Plasma						Liver					
Digitonin-Precipitable Sterols						Digitonin-Precipitable Sterols					
¹⁴ C			Specific Activity			¹⁴ C			Specific Activity		
Per Total Plasma*	Per ml Plasma	% Ester	Total	Free	Ester	Per Whole Organ	Per g Organ	% Ester	Total	Free	Ester
9.9	0.85	60.0	1.58	1.41	1.71	64.4	5.42	60.7	2.58	1.08	23.8
8.5	0.58	55.2	0.70	0.87	0.61	64.8	4.04	57.9	2.02	0.62	16.5
38.1	3.72	76.4	4.73	3.88	5.08	42.0	3.30	61.1	1.52	0.27	>46
53.3	4.91	74.9	8.22	6.17	9.25	24.1	1.67	48.3	0.78	0.18	>19
55.7	6.02	65.1	12.59	10.94	13.70	27.2	2.50	59.5	1.25	0.24	>37
34.2	3.25	63.0	4.67	4.29	4.92	13.5	1.17	56.0	0.62	0.13	>17
19.8	1.89	76.3	3.26	2.30	3.74	35.3	2.85	65.1	1.34	0.23	>44
19.7	1.61	74.7	2.90	2.03	3.39	30.8	2.22	57.9	1.17	0.24	>34
55.6	4.96	58.7	—	—	—	18.9	1.65	51.0	—	—	—
62.7	4.75	63.7	—	—	—	17.6	1.30	55.6	—	—	—
66.5	5.54	65.1	—	—	—	16.7	1.36	52.5	—	—	—
23.4	1.72	61.4	—	—	—	57.1	4.10	64.3	—	—	—
33.7	2.72	66.0	—	—	—	37.3	2.93	56.4	—	—	—
16.3	1.16	62.9	—	—	—	65.0	4.50	63.4	—	—	—

spun for 30 min at 19,800 × g (22). The chylomicrons (*S_f* > 1000) formed a turbid upper layer which was collected with mild suction. When the chylomicron preparation thus obtained was recentrifuged under conditions identical with those used for its preparation from the chyle, 94.3% of its total ¹⁴C was recovered in the *S_f* > 1000 fraction—a finding which attests to the purity of the chylomicron preparation. One milliliter of this preparation was injected intravenously into each recipient rat.

The recipient rat was anesthetized with ether during the intravenous injection of chylomicrons. It was again anesthetized, if necessary, for withdrawal of blood from the abdominal aorta into a heparinized syringe during the 20 sec before it was killed. Immediately after withdrawal the blood was centrifuged for 15 min at 1500 × g at 4°, and the separated plasma was stored at 0° for no longer than 3 hr before analysis. The liver was next excised in about 10 sec from one of the exact times indicated in Table 3. Finally, portions of the mammary glands were sampled at random from areas where maximum density of mammary gland tissue was observed. (In several cases the inner connective tissue membranes were removed together with the entire mammary glands; no attempt was made to separate the glands from the very thin connective tissue linings. The amounts of glandular material excised in this way amounted to 4% of the rats' body weights. This value was used to calcu-

late total ¹⁴C recoveries per whole gland.) The tissues were blotted with filter paper, weighed, and quickly minced with a razor blade.

Plasma, liver, and mammary gland samples were analyzed for sterols by two different methods. (a) Measured portions of these tissues were saponified with a solution of freshly prepared sodium ethylate in ethanol, and the hydrolysates extracted with chloroform (11). Total digitonin-precipitable sterols were isolated, and their amounts and ¹⁴C contents were determined as outlined in section I. (b) Other tissue aliquots were homogenized in a 0.9% NaCl solution and then extracted with chloroform-methanol 2:1 by the method of Folch, Lees, and Sloane Stanley (23). The protein-containing precipitates formed during this extraction procedure were separated by filtration through glass wool and re-extracted twice with the chloroform-methanol mixture. The absence of lipid ¹⁴C in the precipitates after extraction was demonstrated by saponification of the precipitates, extraction of the hydrolysates with chloroform, and counting of the chloroform extracts. The total lipid extracts were evaporated to dryness under reduced pressure, and the residues were dissolved in known volumes of hexane. Duplicate portions of each lipid solution were chromatographed on silicic acid columns (24, 25), and the eluted free and esterified sterol-containing fractions were assayed for ¹⁴C and digitonin-precipitable sterol contents. From these measurements

the specific activities of free and esterified digitonin-precipitable sterols were calculated. Other portions of the lipid extracts were evaporated in counting vials, and their ^{14}C contents were measured. In all cases the values for total ^{14}C and digitonin-precipitable ^{14}C agreed closely. Thus, the injected cholesterol- $4\text{-}^{14}\text{C}$ had not been converted to nondigitonin-precipitable lipids. The values recorded in Table 3 are the averages of those obtained by procedures *a* and *b*.

Results

Three experiments were performed (Table 3). In the first, two lactating rats were killed 11 min after the chylomicron injection. This interval was chosen because Naidoo, Lossow, and Chaikoff (26) found that recirculation of labeled cholesterol did not occur until after that time when very low density chyle lipoproteins containing cholesterol- ^{14}C were injected intravenously. Our 11-min interval, therefore, allowed ample time for various tissues to incorporate the ^{14}C from plasma (resulting in low levels of plasma ^{14}C so that eventual corrections for ^{14}C in plasma included in excised tissues would be small), while the plasma still contained enough ^{14}C for accurate measurement. In this first experiment, 5–11% of the injected ^{14}C was recovered in the lactating mammary gland, about 65% in the liver and about 9% in total plasma (Table 3). A remarkable decrease in the proportion of cholesterol esters occurred in the mammary gland: while the injected chylomicrons contained about 55% of their cholesterol- ^{14}C in the esterified form and this same percentage of ester ^{14}C was recovered from liver and plasma, the mammary gland contained only about 16% of its ^{14}C as esters.

In the second experiment (Table 3) lactating rats were killed 2.5 and 12 min after the chylomicron injection, and postlactating rats 12 min after. In the lactating rats, 12–27% of the injected ^{14}C was recovered in the mammary glands at the 2.5-min interval, and similar percentages—from 17 to 32%—at the 12-min interval. The livers of these rats contained 14–27% of the injected ^{14}C at the early interval, and about 33% at the later one; total plasma contained 34–56% and about 20% of the injected ^{14}C at the two intervals, respectively. Mammary glands of the postlactating rats contained only about 1% of the injected ^{14}C , or 0.11% per gram of gland. Since about 4.5% of the injected ^{14}C was present per milliliter of plasma, the presence of 0.025 ml of plasma in each gram of postlactating mammary gland could account for all of the ^{14}C recovered in that tissue. But in the case of the lactating mammary glands, such a correction for inclusion of plasma ^{14}C in glandular tissue would not appreciably alter the values shown in Table 3.

Accurate determinations of specific activities of esterified sterols in liver and mammary gland were hampered by the fact that only minute amounts of sterol esters were found in these tissues. In Experiment 1, where 15 g of silicic acid-Hyflo Supercel 2:1 was used per column (24), amounts of lipids large enough for accurate measurements of the eluted esterified sterols could be applied only in the case of the livers. In Experiment 2, in which 1-g Unisil columns were used (25), less than 10 μg of sterols were present in the esterified sterol fractions obtained from the maximum amounts of hepatic and glandular lipids that could be applied to each column (about 20 mg of total lipids). However, since these minute amounts of sterol esters contained more than half of the ^{14}C recovered in liver and mammary gland (Table 3), their specific activities must have been relatively high. The specific activity values listed in Table 3 are minimum values estimated on the basis of the highest recoveries of sterol esters observed in the animals studied. Hence, the specific activities of the sterol esters in liver and mammary gland may have been much higher than those recorded in Table 3. It should be noted, in addition, that some of the sterol esters recovered from liver and lactating mammary gland were undoubtedly those of plasma included in these tissues.

In the third experiment, three lactating rats were killed 2 min and three lactating rats 11 min after injection of the labeled chylomicrons. In their mammary glands, 8–29% of the injected ^{14}C was recovered; the ^{14}C recoveries at the early time interval were not lower than those at the later interval, in agreement with Experiment 2. Uptake of chylomicron cholesterol by the lactating mammary gland must therefore proceed very rapidly. In the liver, on the other hand, only 16–19% of the injected ^{14}C was recovered at the 2 min interval and considerably more, 37–65%, at 11 min. It should be noted that in half of the rats studied at the 2 min interval, the lactating mammary gland contained more ^{14}C than did the liver (Table 3).

At the 2 min interval, about 52% of the cholesterol- ^{14}C recovered in the mammary gland was in the esterified form—only slightly less than that in the injected chylomicron ^{14}C —but at 11 min, only 26% of the ^{14}C in the mammary gland was recovered as esterified cholesterol. This decrease in proportion of cholesterol- ^{14}C ester, which was also observed in Experiment 1, may indicate that hydrolysis of the esterified cholesterol- ^{14}C occurred in the mammary gland. Indeed, the presence of a hydrolytically active cholesterol esterase enzyme in the mammary gland was found (unpublished observation). Alternatively, the preponderance of free cholesterol- ^{14}C in the mammary gland at the later time interval could indicate that mostly free cholesterol- ^{14}C is taken up by the gland from the blood stream. However, such a mechanism is

not indicated by the presence of large proportions of esterified cholesterol- ^{14}C in the gland at the 2 min interval in Experiment 3 and at both the 2 and 12 min intervals in Experiment 2. Furthermore, the finding that the specific activity of the esterified cholesterol fraction in the mammary gland was much higher than that in plasma (Table 3) indicates a removal of the high specific activity chylomicron cholesterol esters from plasma by the lactating mammary gland.

Our demonstration that in rats chylomicron cholesterol is taken up directly by the lactating mammary gland from plasma extends earlier observations by Lossow, Brot, and Chaikoff (27), who showed that many extrahepatic tissues are capable of removing cholesterol from very low density chyle lipoproteins. In lactating guinea pigs, too, the mammary gland is capable of directly removing the cholesterol moiety of chylomicrons (28). Furthermore, uptake of chylomicron glycerides during lactation has been demonstrated in the mammary glands of both guinea pigs (2) and goats (3, 4).

In our experiments, 1 ml of the labeled chylomicron suspension was *injected* rapidly into each rat. Therefore, our observations may not reflect the extent to which the lactating mammary gland removes cholesterol for *circulating* chylomicrons under physiological conditions in which chylomicrons enter the circulation continuously. Our experiments do, nevertheless, demonstrate the ability of the lactating mammary gland to remove cholesterol from circulating chylomicrons. The postlactating mammary gland incorporated practically no chylomicron cholesterol, whereas the lactating gland absorbed up to 32% of the injected chylomicron cholesterol within the first 12 min after the injection. At that time, about 20% of the injected ^{14}C was still present in the circulation, largely in the form of very low density lipoproteins (29), and therefore it is likely that the mammary gland would have incorporated even more of the labeled cholesterol had the experimental periods been extended. Furthermore, in view of the rapidity of chylomicron cholesterol removal by the lactating mammary gland, it is conceivable that under physiological conditions, when chylomicrons enter the circulation gradually, the mammary gland takes up a greater proportion of the chylomicron cholesterol than it did when 1 ml of a chylomicron preparation was injected rapidly.

III. EQUILIBRATION OF CHOLESTEROL AMONG PLASMA, LIVER, AND MILK OF RATS FED FOR SEVERAL WEEKS A DIET CONTAINING CHOLESTEROL-4- ^{14}C

Experimental Methods

Preparation of diet. In diet B (Table 1) an aqueous solution of vitamins A and D was used instead of fish oil because the latter contains an unknown amount of cholesterol.

An ether solution was prepared containing 5 g of cholesterol that had been purified by bromination (14) and 50 μC of cholesterol-4- ^{14}C that had been purified by column chromatography (25). The specific activity of the cholesterol in this solution was 11,780 cpm/mg. The ether solution was added dropwise to a thin layer of casein in a porcelain tray, and the ether was allowed to evaporate. All dietary constituents were then mixed mechanically for 24 hr. Five 100-mg samples of the mixed diet were extracted with chloroform-methanol 2:1 and assayed for ^{14}C . Five additional 100-mg samples were treated overnight, each with 1 ml of methanol and 15 ml of the scintillator-containing toluene solution, and their ^{14}C was determined; quenching due to solid particles was corrected for by internal standardization. The ^{14}C contents of all diet samples agreed within 4%—which attests to the homogeneity of the diet—and amounted to 5,340 cpm/g of diet. From the specific activity of 11,780 cpm/mg of added cholesterol, it follows that the diet contained 45 mg of added labeled cholesterol per 100 g. However, skim milk powder also contributed some cholesterol to the diet. In order to determine this contribution, duplicate portions of the skim milk powder were saponified with sodium ethylate solution and analyzed for their cholesterol contents. It was found that 7.5 g of the skim milk powder—the amount in 100 g of diet—contained 1.0 mg of cholesterol. If the dilution of the added labeled cholesterol by the milk powder cholesterol is taken into account, the specific activity of total cholesterol in the diet amounted to 11,500 cpm/mg, and the dietary cholesterol content was 0.046%.

Treatment of Rats. Pregnant, Long-Evans rats with known mating times were used. On the 14th day of pregnancy the rats were placed in individual cages and fed diet B (Table 1) ad libitum until their young were weaned (20 days after birth). A record was kept of their daily food intake (Table 4). Four hours after removal of their litters, the mothers were injected intraperitoneally with one IU of pitocin (Parke, Davis and Co.) and milk was expressed by hand from the nipples. Anesthesia was not used as the mothers seemed not to object to this milking procedure. About 400 μl of milk was obtained from each rat. The rats were then anesthetized with ether, and a 5 ml blood sample was taken from the aorta of each one. The livers were excised, rinsed with 0.9% NaCl solution, blotted with filter paper, and weighed. Finally, mammary gland tissue was removed. From each litter one pup was killed immediately after weaning, and the white material in its stomach was sampled.

Analytical Methods. The blood was centrifuged and the plasma separated. Samples of plasma, liver, mammary gland, milk, and stomach contents were saponified with the sodium ethylate solution, and the specific activities

TABLE 4 SPECIFIC ACTIVITIES OF CHOLESTEROL IN LIVER, PLASMA, MAMMARY GLAND, AND MILK OF RATS FED A DIET CONTAINING CHOLESTEROL-4-¹⁴C FROM 7 DAYS PREPARTUM TO 20 DAYS POSTPARTUM

The specific activity of the dietary cholesterol was 11,500 cpm/mg.

Rat No.	Body Weight*	No. of Litter*	Liver Weight†	Daily Food Intake‡		Specific Activity of Digitonin-Precipitable Sterols in:				Ratio (A)/(B)
				Range	Average	Liver	(B) Plasma	Mammary Gland	(A) Milk	
1	g 290	6	% 5.47	g 25-48	g 34.5	cpm/mg 1860	cpm/mg 1730	cpm/mg 1670	cpm/mg 1450	0.84
2	280	9	6.49	39-47	42.6	1540	1510	1230	1180	0.78
3	308	1	3.70	14-24	18.4	1380	1290	1080	1090	0.84
4	322	2	4.85	26-32	27.8	1820	1710	1520	1500	0.88
5	310	2	5.20	14-39	31.4	1670	1610	1360	1140	0.71
6	296	9	5.70	37-50	45.6	2160	2040	1870	1700	0.83
7	280	7	6.10	27-41	33.1	922	875	914	821	0.94
8	—	6	—	—	—	1810	1720	—	1480	0.86
9	—	6	—	—	—	2000	—	—	1470	0.74§
10	—	6	—	—	—	982	942	—	855	0.91

* On day killed.

† Expressed as per cent of body weight.

‡ Measured during last 2 weeks of lactation.

§ Ratio of specific activities of sterols in milk and liver.

of their digitonin-precipitable sterols were measured as described above.

Results

The results (Table 4) confirm observations reported by Kennedy, Pearce, and Parrott (30, 31) that liver weights and food intakes of lactating rats are positively related to the number of pups in their litters.

In an earlier study (6) in which a diet containing 0.05% cholesterol-4-¹⁴C was fed to rats, the specific activities of cholesterol for a number of tissues, including liver and plasma, attained maximum values in 3 weeks and remained constant with continued feeding of the labeled diet. In that study, when isotopic equilibrium was reached, the specific activity of cholesterol in the liver was only slightly higher than that in plasma. The same phenomenon was observed here (Table 4). Furthermore, a similarity in the specific activities of cholesterol in mammary gland and milk was found (Table 4), which indicates that the isotopic equilibrium extends to the milk-producing system. The specific activities of the stomach contents of the pups agreed closely with that of milk, and therefore are not presented separately. On the average, the specific activity of milk cholesterol was about 83% of that of plasma, and 11% of that of the diet.

IV. DISCUSSION

In an earlier study from this laboratory (6) the origin of a tissue's cholesterol was expressed in terms of the relative contributions by three sources: diet, the liver-plasma pool, and in situ synthesis. In that study it was assumed that the liver processed all ingested labeled cholesterol and that it was the principal or only source of

endogenously synthesized plasma cholesterol. If the results of the present equilibration study were interpreted in this way, our finding that, at isotopic equilibrium, the specific activity of milk cholesterol was about 11% of that of the diet and about 83% of that of plasma would indicate the following contributions to the milk cholesterol: 11% from the diet, 16% from synthesis in the mammary gland, and 73% from the liver-plasma pool.

We now question this earlier interpretation of equilibrium studies because it was based on the assumption that at isotopic equilibrium all plasma cholesterol is uniform in its specific activity. It fails to take into account that, even at isotopic equilibrium, labeled dietary cholesterol of considerably higher specific activity than that of plasma, entering the blood stream in the form of chyle lipoproteins, is rapidly taken up not only by liver but also by a number of extrahepatic tissues (27), including the lactating mammary gland (see above).

Obviously, our objection to the earlier interpretation of cholesterol-¹⁴C feeding experiments does not apply to the calculation of the exogenous cholesterol contribution to the milk: since ingested cholesterol was diluted in the body of the rat to 11% of its original specific activity before being excreted in milk, 11% of the milk cholesterol must have been of dietary origin and the remainder, 89%, endogenously synthesized. But an important question now arises: in which form was the dietary cholesterol taken up by the mammary gland—(a) as a liver-treated lipoprotein in which the cholesterol specific activity is low due to dilution with liver cholesterol, or (b) as chylomicrons in which cholesterol is of high specific activity? The relative participations of processes (a) and (b) in transporting dietary cholesterol to the mammary gland determine the extent to which the

endogenous cholesterol sources contribute to milk, as shown by the following calculation.

An Estimate of the Daily Cholesterol Contribution by Chylomicrons to Milk

The food intake of the lactating rats in the cholesterol-4-¹⁴C-feeding experiment (Table 4) averaged 33.3 g per rat per day. Since the diet contained 0.046% cholesterol with a specific activity of 11,500 cpm/mg, 15.3 mg of cholesterol with a total radioactivity of 176×10^3 cpm was ingested (on the average) per rat per day.

Only a fraction of the ingested cholesterol is absorbed. To measure the extent of absorption, three normal female rats and one lactating rat nursing five pups were made to swallow a gelatin capsule containing about 0.4 μ mole (5 μ c) of cholesterol-4-¹⁴C. Thereafter, the rats were placed in individual metabolism cages where they had free access to Purina Lab Chow and water. The feces, collected for 3 days, were homogenized in chloroform-methanol 2:1 in a Waring Blendor, extracted, and assayed for total digitonin-precipitable ¹⁴C. From 23 to 32% (average 28%) of the ingested ¹⁴C was recovered from the feces, the percentage being the same for the lactating and nonlactating rats. Because part of the digitonin-precipitable ¹⁴C-sterols recovered from the feces represented cholesterol-¹⁴C that had reentered the intestine via the enterohepatic circulation and because part of the digitonide ¹⁴C may have been coprosterol-¹⁴C, 28% is probably a maximum estimate of unabsorbed cholesterol-¹⁴C. We may therefore conclude that at least 72% of the cholesterol ingested per day was absorbed, amounting to 11.0 mg containing 127×10^3 cpm. A similar degree (percentage) of cholesterol absorption was found by both Chevallier (5) and Wilson (32) in experiments in which diets containing 0.05% cholesterol were fed to rats.

Absorbed cholesterol is diluted with intestinal cholesterol during its passage into chyle. Swell, Trout, Hopper, Field, and Treadwell (33, 34) found, in rats, a dilution of one part of intestinal cholesterol between the 4th and 12th hour postprandially. We have assumed that our lactating rats ate more often than twice per day, so that Swell's chyle data, obtained from rats in the postabsorptive state for less than 12 hr, are applicable to our cholesterol-4-¹⁴C-feeding experiment. However, in our experiment the dietary cholesterol is mixed with labeled intestinal cholesterol. From the work of Morris and Chaikoff (6) it can be calculated that after feeding a diet containing 0.05% cholesterol-4-¹⁴C until isotopic equilibrium is reached, the specific activity of intestinal cholesterol is about 70% of that of the liver. In the present experiment, the average specific activity of liver cholesterol was 1,615 cpm/mg (Table 4); hence, the specific

TABLE 5 SPECIFIC ACTIVITIES OF CHOLESTEROL IN CHYLE, LIVER, AND BLOOD OF RATS FED A DIET CONTAINING CHOLESTEROL-4-¹⁴C FOR 3 WEEKS

The specific activity of the dietary cholesterol was 11,500 cpm/mg.

Rat No.	Body Weight	Specific Activity of Digitonin-Precipitable Sterols in:		
		Chyle*	Liver	Blood†
	g	cpm/mg	cpm/mg	cpm/mg
1	215	4170	2210	2100
2	205	4280	2560	2390

* Collected from the thoracic lymph duct during first 2 hr after cannulation.

† Obtained at time rat was killed.

activity for intestinal cholesterol was about 1,140 cpm/mg. When the 11.0 mg (127×10^3 cpm) of absorbed dietary cholesterol was mixed with an equal amount of intestinal cholesterol with a specific activity of 1,140 cpm/mg, 22.0 mg of cholesterol (139×10^3 cpm) was passed into the intestinal chyle per day.

Practically all dietary cholesterol absorbed into the chyle is transported in the form of very low density lipoproteins ($S_f > 20$), with more than 90% as chylomicrons ($S_f > 1000$) (35, 36). In our experiment, 22.0 mg of cholesterol containing 139×10^3 cpm and thus having a specific activity of about 6,330 cpm/mg enters the blood stream daily in the form of these very low density chyle lipoproteins. The specific activity of cholesterol in these chyle lipoproteins is thus about four times higher than that in plasma at isotopic equilibrium (Table 4); this is not surprising since, so long as the body synthesizes unlabeled cholesterol, the specific activity of the body cholesterol—including that of plasma—must be lower than that of the incoming chyle, even at isotopic equilibrium. The finding of Morris, Chaikoff, Felts, Abraham, and Fansah (37) that about 75% of plasma cholesterol is of endogenous origin when rats are fed a diet containing 0.05% cholesterol is in perfect agreement with the above result that chyle lipoprotein cholesterol which enters the blood stream must have a specific activity about four times higher than that of plasma at isotopic equilibrium. Further evidence for this was obtained by feeding two normal rats a diet containing 0.5% cholesterol-4-¹⁴C (diet B in Table 1) for 3 weeks and comparing at isotopic equilibrium the specific activities of cholesterol isolated from livers, blood, and thoracic chyle. Table 5 shows that the specific activity of cholesterol in *whole chyle*⁴ was about double that in

⁴ Just enough chyle (about 1 ml) was collected for an accurate cholesterol determination. Because flow of chyle is slow shortly after thoracic duct cannulation, the collection time was about 2 hours. Conceivably, collecting for a longer period—to obtain enough chyle for a cholesterol determination in chylomicrons—would have disturbed isotopic equilibrium in the donor rat.

blood. This means that the specific activity of *chylomicron* cholesterol exceeded that of blood cholesterol by a factor of more than two, since dietary cholesterol is transported mainly by chylomicrons (35, 36).

In Section II it was shown that the lactating mammary gland incorporated 5–32% (average 16%) of the ^{14}C from the intravenously-injected, cholesterol-4- ^{14}C -labeled chylomicrons within the first 12 min after the injection, and most of it during the first 2 min. When we apply the data obtained from the chylomicron-injected rat to the cholesterol-4- ^{14}C -fed rat, 16% of the cholesterol that enters the blood stream in the form of the very low density chyle lipoproteins (i.e., 3.5 mg of cholesterol containing 22,300 cpm/day) is taken up by the lactating mammary gland. Since the rats were in isotopic equilibrium, at least the same amount of labeled cholesterol as entered the mammary gland in the form of chyle cholesterol was excreted by the gland into the milk—provided no labeled cholesterol was returned by the gland to plasma.

An Estimate of the Daily Cholesterol Excretion into Milk

The daily milk production of rats cannot be estimated by simple milking techniques (38). In a carefully designed study, Brody and Nisbet (39) reported that rats weighing 300 g and nursing six pups produce on the 16th day of lactation an average of about 26 ml of milk. The average cholesterol content of rat milk observed in the present study was 0.65 mg/ml, a value in good agreement with that reported by Okey, Godfrey, and Gillum (40). Thus, 26×0.65 mg of cholesterol with an average specific activity of about 1270 cpm/mg (Table 4) was excreted into milk per day, i.e., 16.0 mg of cholesterol containing 21,500 cpm.

V. CONCLUSIONS

About 11% of the milk cholesterol is of dietary origin, as judged from the ratio of the specific activities of cholesterol in milk and diet at isotopic equilibrium. The form in which this dietary cholesterol is taken up by the mammary gland determines the proportionate contributions of cholesterol by endogenous sources—liver and mammary gland—to milk. If it is assumed, in keeping with an earlier interpretation of isotope equilibration experiments (6), that all dietary cholesterol- ^{14}C is first processed by the liver before it becomes available to extrahepatic tissues, our data indicate that the liver is the principal endogenous source of milk cholesterol. On the other hand, uptake by the mammary gland of dietary cholesterol- ^{14}C in the form of low density chyle lipoproteins, estimated above to be about 3.5 mg of cholesterol containing 22,300 cpm, could readily account for all the cholesterol- ^{14}C (21,500 cpm) and for about

20% of the total cholesterol (about 17 mg) excreted daily into milk. This means that the liver, since it was labeled, could not have contributed cholesterol to milk (apart from a possible cholesterol contribution by liver to small intestine, where dietary and endogenous cholesterol mix), and hence, that about 80% of the milk cholesterol was produced *de novo* in the mammary gland.

It is not our intention to propose that dietary cholesterol contributes to milk *exclusively* in the form of chyle lipoproteins. Although that possibility cannot be excluded on the basis of our data, it leads to the unlikely conclusions that the mammary gland, which showed a low activity in incorporating labeled precursors into sterols *in vitro* (Table 2), produced about 80% of the milk cholesterol and that the liver did not contribute cholesterol to the milk. It is probable, however, that a portion of the dietary cholesterol processed by the liver and mixed with liver cholesterol contributes to milk. Lack of information on the extent to which cholesterol from the very low density chyle lipoproteins is taken up by mammary gland and liver *under physiological conditions* precludes a definite assessment of these two endogenous sources of milk cholesterol. Indeed, lack of information regarding uptake of chyle lipoproteins by various tissues under physiological conditions casts doubt on the quantitative interpretability of cholesterol- ^{14}C -feeding experiments reported in the literature (5, 6).

The technical assistance of Miss Sandra K. Stoddard is gratefully acknowledged.

This investigation was supported by grants from the U. S. Public Health Service and the Sacramento County Heart Association.

Manuscript received 27 May 1965; accepted 23 August 1965.

REFERENCES

1. Folley, S. J. In *The Physiology and Biochemistry of Lactation*. Oliver and Boyd, London, 1956, p. 93.
2. McBride, O. W., and E. D. Korn. *J. Lipid Res.* **5**: 453, 1964.
3. Barry, J. M., W. Bartley, J. L. Linzell, and D. S. Robinson. *Biochem. J.* **89**: 6, 1963.
4. Storry, J. E., and J. A. Rook. *Biochem. J.* **91**: 27C, 1964.
5. Chevallier, F. *Biochim. Biophys. Acta* **84**: 316, 1964.
6. Morris, M. D., and I. L. Chaikoff. *J. Biol. Chem.* **234**: 1095, 1959.
7. Krebs, H. A., and K. Henseleit. *Z. Physiol. Chem.* **210**: 33, 1932.
8. McIlwain, H., and H. L. Buddle. *Biochem. J.* **53**: 412, 1953.
9. Baruch, H., and I. L. Chaikoff. *Proc. Soc. Exptl. Biol. Med.* **86**: 97, 1954.
10. Entenman, C., S. R. Lerner, I. L. Chaikoff, and W. G. Dauben. *Proc. Soc. Exptl. Biol. Med.* **70**: 364, 1949.
11. Hotta, S., R. Hill, and I. L. Chaikoff. *J. Biol. Chem.* **206**: 835, 1954.

12. Sperry, W. M., and M. Webb. *J. Biol. Chem.* **187**: 97, 1950.
13. Zlatkis, A., B. Zak, and A. J. Boyle. *J. Lab. Clin. Med.* **41**: 486, 1953.
14. Fieser, L. F. *J. Am. Chem. Soc.* **75**: 5421, 1953.
15. Schwenk, E., G. J. Alexander, and C. A. Fish. *Arch. Biochem. Biophys.* **58**: 37, 1955.
16. Schwenk, E., D. Todd, and C. A. Fish. *Arch. Biochem. Biophys.* **49**: 187, 1954.
17. Abraham, S., and I. L. Chaikoff. *J. Biol. Chem.* **234**: 2246, 1959.
18. Hirsch, P. F., H. Baruch, and I. L. Chaikoff. *J. Biol. Chem.* **210**: 785, 1954.
19. Balmain, J. H., S. J. Folley, and R. T. Glascock. *Biochem. J.* **52**: 301, 1952.
20. Abraham, S., I. L. Chaikoff, and P. Cady. *Cancer Res.* **21**: 938, 1961.
21. Bloom, B., I. L. Chaikoff, W. O. Reinhardt, C. Entenman, and W. G. Dauben. *J. Biol. Chem.* **184**: 1, 1950.
22. Hillyard, L. A., C. Entenman, H. Feinberg, and I. L. Chaikoff. *J. Biol. Chem.* **214**: 79, 1955.
23. Folch, J., M. Lees, and G. H. Sloane Stanley. *J. Biol. Chem.* **226**: 497, 1957.
24. Barron, E. J., and D. J. Hanahan. *J. Biol. Chem.* **231**: 493, 1958.
25. Creech, B. G., and B. Sewell. *Anal. Biochem.* **3**: 119, 1962.
26. Naidoo, S. S., W. J. Lossow, and I. L. Chaikoff. *J. Lipid Res.* **3**: 309, 1962.
27. Lossow, W. J., N. Brot, and I. L. Chaikoff. *J. Lipid Res.* **3**: 207, 1962.
28. McBride, O. W., and E. D. Korn. *J. Lipid Res.* **5**: 459, 1964.
29. Lossow, W. J., S. S. Naidoo, and I. L. Chaikoff. *J. Lipid Res.* **4**: 419, 1963.
30. Kennedy, G. C., W. M. Pearce, and D. M. V. Parrott. *J. Endocrinol.* **17**: 158, 1958.
31. Kennedy, G. C. *Proc. Roy. Soc., Ser. B.* **140**: 578, 1952.
32. Wilson, J. D. *J. Lipid Res.* **5**: 409, 1964.
33. Swell, L., E. C. Trout, Jr., J. R. Hopper, H. Field, Jr., and C. R. Treadwell. *J. Biol. Chem.* **232**: 1, 1958.
34. Swell, L., E. C. Trout, Jr., J. R. Hopper, H. Field, Jr., and C. R. Treadwell. *J. Biol. Chem.* **233**: 49, 1958.
35. Hillyard, L. A., I. L. Chaikoff, C. Entenman, and W. O. Reinhardt. *J. Biol. Chem.* **233**: 838, 1958.
36. Byers, S. O., and M. Friedman. *Am. J. Physiol.* **179**: 79, 1954.
37. Morris, M. D., I. L. Chaikoff, J. M. Felts, S. Abraham, and N. O. Fansah. *J. Biol. Chem.* **224**: 1039, 1957.
38. Luckey, T. D., T. J. Mende, and J. Pleasants. *J. Nutr.* **54**: 345, 1954.
39. Brody, S., and R. Nisbet. *Mo. A. E. S. Res. Bull.* **285**: 5, 1938.
40. Okey, R., L. S. Godfrey, and F. Gillum. *J. Biol. Chem.* **124**: 489, 1938.
41. Cartland, G. F., and F. C. Koch. *Am. J. Physiol.* **85**: 540, 1928.