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Myocardial Metabolism IV. Metabolism of Free and Esterified Cholesterol by the Perfused Rat Heart and Homogenates* (34026)

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It is well documented (1) that circulating fatty acids provide an important energy source to the myocardium, and several studies have been directed to the importance of albumin-bound free fatty acids in this respect (2–5). More recent investigations have extended the early observation of Bing *et al* (6) which indicated that a more important source of fatty acids was derived from circulating esterified lipids which comprise over 90% of the total circulating lipid. These studies (7–12) have revealed that rabbit and rat heart can extract and oxidize triglyceride fatty acid from density < 1.006 lipoproteins; this was also the case for triglyceride of the density 1.006–1.070 lipoproteins, but to a lesser extent (9). Phospholipids from all lipoprotein fractions were not extracted by isolated perfused rat heart and were, therefore, not an energy source to this tissue (9).

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Miller *et al.* (13) also reported that during perfusion of rabbit hearts with hypercholesteremic plasma, there was an increase in circulating free cholesterol with a proportional loss in esterified cholesterol. These studies were carried out under hypoxic conditions and, when oxgenated red blood cells were added to the perfusing diluted serum, less dramatic results were obtained. Also, Delcher *et al.* (9) were not able to ascertain whether the perfused rat heart could extract or utilize circulating free and esterified cholesterol.

It was the purpose of the present study to determine whether (1) the isolated perfused rat heart is able to extract labeled cholesterol and cholesterol palmitate; (2) cholesterol ester fatty acid is oxidized; (3) heart contains cholesterol ester hydrolase and/or low- or high-energy cholesterol-esterifying system; and (4) the extent of cholesterol biosynthesis from labeled mevalonate.

Materials and Methods. Cholesterol-4-^{14}C,

cholesterol palmitate-1-^{14}C, sodium acetate-2-^{14}C, and mevalonic acid-2-^{14}C-lactone were obtained from Nuclear Chicago Corp. Cholesterol and cholesterol palmitate were purchased from Hormel Institute and other chemicals were from Calbiochem (California) or Fisher Scientific Co. Bovine serum albumin, fraction V (Nutritional Biochemicals) was used without further purification.

Male albino rats of the Wistar strain (Microbiological Associates) were maintained on a stock diet *ad libitum* prior to use. The animals were anesthetized with intraperitoneal pentobarbital before exposure of the thoracic cavity and removal of the heart.

The perfusion medium consisted of modified Krebs-Henseleit bicarbonate buffer (14), pH 7.4, containing 150 mg/100 ml glucose and 2.5% bovine serum albumin, Fraction V. The perfusion apparatus and procedure for aortic perfusion in an open recirculation system have been described in detail earlier (15). The perfusate was sampled initially and at 15-min intervals for determination of substrate uptake and $^{14}CO_2$ production. At the end of perfusion, the heart and cannula were removed from the apparatus, 5 ml of Krebs buffer were forced through the coronaries to remove labeled substrate. The hearts were blotted, weighed, and homogenized in 20 vol of chloroform-methanol (2:1). Aliquots of the perfusion medium were also placed in chloroform-methanol and these were extracted according to Folch *et al* (16).

Samples of the perfusate (0.5 ml) and of the NaOH trap (1 ml) were acidified with 5 ml 6 *N* H_2SO_4, and $^{14}CO_2$ was trapped in hyamine hydroxide as described by Cuppy and Crevasse (17). These were counted in a Nuclear-Chicago liquid scintillation spectrometer (model 720) using 10 ml of toluene scintillant mixture containing 2,-5-diphenyloxazole (4 g/liter) and *p*-bis-2′-(5′ phenyloxazolyl) benzene (500 mg/liter).

After extraction of the heart and perfusate lipids (16), aliquots of the extract were counted and samples fractionated by thin-layer silicic acid chromatography using a solvent system of hexane:acetone:acetic acid (90:11:3). The lipids were visualized by exposure to iodine vapor and areas corresponding to authentic lecithin, monoolein, diolein, oleic acid, triolein, cholesterol, and cholesterol oleate were individually scraped into scintillation vials. One milliliter of methanol and 10 ml scintillant were added prior to counting.

Cholesterol biosynthesis by heart and liver homogenates *in vitro* were studied using the conditions described by Popjak *et al.* (18), which are summarized in Table IV. The tissue homogenates in 0.12 *M* sucrose (19) were centrifuged at 10,000*g* for 25 min. The reaction mixture containing 4 ml supernatant enzyme and 2 ml substrate mixture was incubated aerobically for 2 hr at 37°. The mixture was hydrolyzed, extracted with petroleum ether, carrier cholesterol was added, and the sterol was precipitated with digitonin according to Sperry and Webb (20). The sterol digitonide was dissolved in methanol for liquid scintillation counting. The recovery of authentic cholesterol-4-^{14}C carried through the procedure was 93–97%.

For assay of hydrolyzing cholesterol esterase activity, 10% homogenates of rat heart were prepared in 0.154 *M* phosphate buffer, pH 6.6. The substrate system contained cholesterol-4-^{14}C oleate in a micellar medium (21). The assay procedure has been described elsewhere (22).

Assays for enzymatic synthesis of cholesterol ester were carried out in two systems. In the first, cholesterol was dispersed in a medium containing albumin, sodium taurocholate, and oleic acid in 0.154 *M* phosphate buffer, pH 6.2, as described elsewhere (22). Heart was homogenized in 4 vol of 0.154 *M* phosphate buffer, pH 6.2, physiological saline, or 50% glycerol in water. After centrifugation at 5000*g* for 10 min, the supernatant fraction was used for assay of cholesterol esterase activity as previously described (22).

The presence of a high-energy-requiring cholesterol esterifying system was determined using the system described for liver (23). Incubations were carried out for 2 hr at 37° and the reaction was terminated by addition

TABLE I. Myocardial Uptake of Free and Esterified Cholesterol during Perfusion *in Vitro*.

Perfusion medium[a]	Myocardial uptake (%)
Cholesterol-4-^{14}C	34.6 ± 2.4 (2)[b]
Cholesterol-4-^{14}C palmitate	48.9 ± 4.6 (9)
Thoracic duct lymph (diluted 1:10) containing 87.4% esterified cholesterol-4-^{14}C	4.8 ± 0.5 (4)

[a] The basic perfusion medium consisted of Krebs bicarbonate buffer, pH 7.4, containing 150 mg/100 ml glucose and 2.5% bovine albumin, fraction V. In the case of the lymph sample, rat lymph was diluted 1:10 with bicarbonate buffer, pH 7.4, prior to perfusion.

[b] Values represent means ± SEM. The number of determinations are shown in parentheses.

of 20 vol of chloroform–methanol (2:1 v/v). After extraction (16), the lipids were separated by thin-layer chromatography and counted as described above.

Results and Discussion. As pointed out by Delcher *et al.* (9) in 1965, circulation of labeled lipids in isolated lipoproteins through tubing and filters of the heart perfusion apparatus resulted in large losses of label (25–38%) despite the absence of a heart. Corrections were made for the losses in each experiment. In the present study, similar losses (55%) were found by circulation of the labeled sterol through the apparatus, and these were due primarily to the filter. Therefore, in the reported studies, the perfusion apparatus was used without the filter.

In the initial studies, cholesterol (70 mg) or cholesterol ester (10–30 mg) were added in ether to 100 ml of Krebs bicarbonate buffer of rat plasma, the ether was evaporated, and the mixture was sonicated at 20° to give a uniform dispersion. However, perfusion of any of these preparations caused cessation of heart beat. Similarly, the use of Tween 20 as a dispersing agent was unfavorable for heart perfusion. Thus, all experiments were performed using tracer amount of labeled cholesterol and cholesterol palmitate or diluted rat lymph labeled with cholesterol.

Between 35 and 50% of the circulating free cholesterol and cholesterol palmitate were extracted by perfused rat heart in 45 min (Table I). During the perfusion, there were no changes in the percentages of free or esterified cholesterol in the perfusion medium (Table II), and analysis of distribution of label in the heart showed that, of the sterol extracted, there was no detectable synthesis or hydrolysis of cholesterol esters.

A 6-hr sample of thoracic duct lymph was obtained (24) after intragastric administration of an aqueous emulsion containing cholesterol-4-^{14}C, oleic acid, sodium taurocholate, and albumin. This was diluted with 9 vol of Krebs bicarbonate buffer and used immediately for perfusions. Initially, 87.4% of the labeled cholesterol in lymph was in the esterified form, and during 45 min of perfusion, there was no hydrolysis of this ester in the circulation medium (Table II). About 5% of the labeled cholesterol was recovered

TABLE II. Isotope Distribution of Perfusion Medium and Heart during Perfusion *in Vitro*.

Perfusion medium	Medium, min of perfusion				Heart
	0	15	30	45	
% Cholesterol unesterified					
Cholesterol-4-^{14}C	99.3	98.1	99.5	99.4	99.8
% Cholesterol as ester					
Cholesterol palmitate-1-^{14}C + lecithin (5 mg/100 ml)	92.8	93.7	97.2	91.0	95.6
Cholesterol palmitate-1-^{14}C + sodium taurocholate (1.67 mg/100 ml)	96.4	96.9	96.4	95.6	96.5
Thoracic duct lymph containing 87.4% esterified cholesterol-4-^{14}C esters	87.4 ±0.3	87.1 ±0.5	87.3 ±0.3	86.9 ±0.6	80.0 ±0.9

TABLE III. Distribution of Radioactivity in Lipid Fractions of Perfusate and Heart during Perfusion with Cholesterol Palmitate-1-^{14}C.[a]

Lipid fraction	Radioactivity, % of total		
	Perfusate		Heart
	0 min	45 min	45 min
Phospholipid–monoglyceride	0.274 ± 0.06	0.180 ± 0.04	0.31 ± 0.01
Diglyceride	0.270 ± 0.04	0.484 ± 0.08	0.37 ± 0.02
Fatty acid	1.50 ± 0.11	1.13 ± 0.09	0.94 ± 0.03
Triglyceride	0.936 ± 0.05	0.904 ± 0.05	1.35 ± 0.09
Cholesterol ester	96.9 ± 0.2	97.3 ± 0.1	97.0 ± 0.1

[a] Values represent the mean ± SEM.

in the perfused heart and of this, 80% was still in the esterified form.

In the studies of Delcher *et al.* (9), esterified cholesterol represented about 1% of the lipid radioactivity in $d < 1.006$ lipoproteins of rat serum. After 30-min heart perfusions, esterified cholesterol represented 1% of the labeling in the heart. These samples were labeled *in vivo* by injection of palmitic acid-1-^{14}C and the extent of uptake and metabolism of cholesterol and its esters could not be ascertained.

The data in Table III summarize the results of perfusions with cholesterol palmitate-1-^{14}C. As in the previous studies, the percentage of esterified sterol remained constant in the perfusate throughout 45 min of perfusion and was not altered in the heart. Monitoring of $^{14}CO_2$ production during this period revealed that no metabolism of the labeled esterified palmitate had occurred.

For studies on cholesterol ester hydrolyzing activity in heart (cholesterol esterase, E.C. 3.1.1.13), a 5000*g* supernatant of heart homogenates was used, and it was not possible to show hydrolysis of the substrate, cholesterol oleate, at either pH 6.6 or 7.4; this was the case whether the substrate was prepared in micellar form or dispersed on albumin (22). Similarly, attempts to show classical cholesterol ester synthesizing activity (22) were without success. In addition to the low energy cholesterol esterase systems of pancreas and intestine, tissues such as liver and adrenal contain a cholesterol esterifying system dependent on the presence of ATP and CoA, or fatty acyl CoA (acyl CoA cholesterol-O-acyl-transferase, EC 2.3.1). Incubation of 20% homogenates of rat heart with the high energy substrate system did not result in formation of cholesterol esters. Thus, it appears that if there is enzymatic synthesis or hydrolysis of cholesterol esters in heart, the substrate requirements or conditions differ from those of the pancreas and liver systems.

Cholesterol biosynthesis from mevalonic acid-2-^{14}C lactone was investigated *in vitro* using assay conditions described by Popjak *et al.* (18), and 10,000*g* supernatant enzymes from liver and heart. Liver from rats fed *ad libitum* incorporated 50 times as much mevalonate into digitonin-precipitable sterols as did livers from rats fasted 48 hr. These data are comparable to those of Dietschy and Siperstein (25) who reported at 15-fold difference using acetate-2-^{14}C. Altering the levels of ATP or reduced pyridine nucleotides did not significantly affect sterol biosynthesis, whereas substitution of aerobic conditions with N_2 atmosphere virtually eliminated mevalonic incorporation into sterol. However, even under optimal conditions for sterol biosynthesis in the liver, it was not possible to show mevalonate incorporation into sterols using heart tissue in several trials. It has been reported that all muscle, including heart, incorporate acetate into sterols at less than 0.6% of the hepatic rate (25).

Finally, the intact myocardium was perfused for 45 min with either 1 μmole mevalonic acid-2-^{14}C lactone or 5 mmoles sodium acetate-2-^{14}C. Again there was no evidence for production of digitonin-precipitable

TABLE IV. Incorporation of Mevalonic Acid-2-^{14}C-Lactone into Digitonin-Precipitable Sterols of Liver and Heart.

Enzyme preparation	Incubation conditions[a]	Mevalonic acid-2-^{14}C-lactone incorporated in 2 hr (mμmoles)
Liver, boiled	Air	0
Liver, 48-hr fasted rat	Air	8
Liver, *ad libitum* fed rat	Air	406
Liver, *ad libitum* fed rat	Air, 2.5 μmoles ATP	416
Liver, *ad libitum* fed rat	Air, 4 μmoles NADH 4 μmoles NADPH	386
Liver, *ad libitum* fed rat	N_2 atmosphere	8
Liver, *ad libitum* fed rat	O_2 atmosphere	394
Heart, 48-hr fasted rat	Air	0
Heart, *ad libitum* fed rat	Air	0

[a] Incubations were carried out by addition of 4 ml enzyme (supernatant after 10,000*g* centrifugation for 25 min) to 2 ml substrate. The substrate with no modifications contained: 2 μmoles *d,l*-mevalonic acid-2-^{14}C-lactone (1 μCi), 2 μmoles NADH, 2 μmoles NADPH, 5 μmoles ATP, and 10 μmoles reduced glutathione. Flasks were incubated for 2 hr in air at 37°.

sterols from these labeled precursors. Evidence from the present study emphasizes the low levels of free and esterified cholesterol metabolism in cardiac tissue and suggests that a large portion of mycardial sterol is used for structural purposes.

Summary. Several aspects of the myocardial metabolism of cholesterol and its esters have been investigated using isolated perfused rat heart and heart homogenates. Between 35–50% of tracer amounts of circulating cholesterol-4-^{14}C and cholesterol palmitate-1-^{14}C were extracted by the heart during 45-min perfusion, but there was no indication of cholesterol ester synthesis or hydrolysis either in the medium or in the heart. Similar studies using heart homogenates revealed the lack of detectable enzymatic hydrolysis of cholesterol oleate and absence of both low- and high-energy–requiring ester synthesizing systems. Further evidence for the inability of heart tissue to split sterol esters was based on the finding that $^{14}CO_2$ was not produced from the palmitate-1-^{14}C moiety of the ester. When diluted rat lymph containing 87% cholesterol-4-^{14}C ester was perfused, about 5% of the circulating label was recovered in the myocardium, and of this, 80% was still esterified sterol.

Hepatic and myocardial cholesterol biosynthesis was investigated using mevalonic acid-2-^{14}C lactone as precursor. Under conditions of optimal biosynthesis of cholesterol by liver homogenates, there was no detectable labeling in digitonin-precipitable sterols in heart homogenates. The present studies suggest that the low levels of myocardial cholesterol metabolism are probably related to structural requirements of this tissue and that the heart plays little or no role in regulating circulating sterol levels.

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Rapid Electrophoretic Examination of Diaphorases in *Mycobacterium tuberculosis* (34027)

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Although diaphorases occur widely in nature, their role in respiration remains obscure. Renewed interest in this area resulted from the finding that extracts of *Mycobacterium tuberculosis* which were resistant to isoniazid contained greater NADH-diaphorase activity than did extracts of drug-susceptible *M. tuberculosis* (1). In the course of these studies a technique was developed which allowed rapid visualization of diaphorases in cell extracts after electrophoretic separation on cellulose acetate membranes. The present report is concerned with describing this technique in detail and illustrating its application to the study of specific tetrazolium reductases.

Methods. *Mycobacterium tuberculosis* H37Rv was maintained by weekly transfer in Middlebrook's 7H10 OA liquid medium. Large numbers of cells were obtained by growth in fermentors containing 14 liters of the same medium. All incubations were carried out at 37°. Cells were harvested while in the logarithmic phase of growth by aerosol-free centrifugation.

In the preparation of cell extracts, recovered cells were washed and made up in thick suspension with Tris buffer, 0.05 *M*, pH 7.0. The suspension was sonicated at icewater temperature for three 10-min intervals with a 5-min rest between each treatment. The sonicate was centrifuged first at 20,000*g* for 30 min and then at 105,000*g* for 90 min. The supernatant fraction from the high-speed centrifugation was removed and filtered through a 0.22-μ filter. This filtrate constituted the cell extract used for all assays. Protein was determined by the Lowry method.

Diaphorases were measured by coupling the oxidation of NADH or NADPH with the reduction of *p*-nitrobluetetrazolium and measuring the increase in absorbancy at 540 mμ. Electrophoretic examination of the extracts was carried out with Sepraphore III as the support medium. Strips were subjected to 450 V for 50–60 min at 5° employing Gelman's high-resolution buffer. Diaphorases were visualized by overlaying the strip with a similar Sepraphore III strip saturated with NADH, buffer, and *p*-nitrobluetetrazolium.