Glucose: a possible intermediate in the oxidation of the side chain of cholesterol in resting and stimulated rats

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ABSTRACT The effect of repeated muscular contraction on the rate of oxidation of the side chain of cholesterol was studied in anesthetized rats. The animals received an intravenous pulse-label injection of either cholesterol-26-^{14}C, incorporated into rat plasma lipoproteins, or bicarbonate-^{14}C. In half the animals of each group, the hind legs were repeatedly stimulated by electrical impulses. A multicompartmental analysis was attempted, based on the disappearance curve of plasma free cholesterol-^{14}C and on the excretion rate of expired $^{14}CO_2$, as well as on previously reported rates of bile acid and adrenal steroid secretion. The rate of expired $^{14}CO_2$ originating from cholesterol-26-^{14}C was much less than that predicted by the digital computer analysis; cholesterol degradation could not be evaluated since the data were incompatible with a model that assumes direct oxidation of the side chain to CO_2. A revised model was postulated in which an important fraction of the side chain of cholesterol would be converted to CO_2 only after previous conversion to glucose. Direct measurement of plasma glucose-^{14}C after the injection of cholesterol-26-^{14}C supported this hypothesis.

SUPPLEMENTARY KEY WORDS cholesterol pools · plasma free cholesterol · plasma esterified cholesterol · irreversible disposal · side-chain oxidation · fractional turnover · multicompartmental model · muscular stimulation · exercise · cholesterol-26-^{14}C · bicarbonate-^{14}C · respiratory $^{14}CO_2$

The degradation of cholesterol as measured by the oxidation of its side chain to expired CO_2 (1, 2) appears to be markedly increased by exercise (3, 4) and by repeated contractions of skeletal muscle (5–7). However, the conclusions of studies based solely on the respiratory excretion of $^{14}CO_2$ after the injection of cholesterol-26-^{14}C must be interpreted with caution (6), since they do not take into account the dilution of labeled materials in intermediate pools. Detailed studies of the conversion of other ^{14}C-labeled substances to $^{14}CO_2$ have been reported for various metabolites, such as glucose (8), fatty acids (9), and mevalonic acid (10). However, except for Myant and Lewis's (2) study of cholesterol-26-^{14}C oxidation to CO_2, in which a two-pool model (cholesterol–respired CO_2) is tacitly assumed to be the basis for calculation, no detailed analysis of oxidation of cholesterol to CO_2 has been reported. The need for such an analysis is especially obvious when one is studying the probable effects of exercise or muscular contraction on cholesterol degradation, since under these conditions both the intermediate and the body bicarbonate–CO_2 pools may have drastically altered sizes and turnover rates. These changes could, in turn, produce marked differences in respiratory $^{14}CO_2$ excretion independent of any actual changes in the rates of cholesterol degradation.

In the present paper, we describe an attempted analysis of cholesterol degradation based upon a model that assumes the direct oxidation of the cholesterol side chain to CO_2. The model proved to be incompatible with the data obtained and with previously estimated rates of bile acid and adrenal steroid formation. However, the data were compatible with a model in which a significant fraction of the side chain of cholesterol is converted to glucose before being oxidized to CO_2.

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MATERIAL AND METHODS

Preparation of Animals

Adult male Sprague-Dawley rats, maintained on Purina Laboratory Chow and water, were used throughout. The methods were essentially similar to those already reported (5–7). Cholesterol-26-^{14}C-labeled plasma lipoproteins were prepared with minor modifications (6). Whole blood obtained from the abdominal aorta of rats anesthetized with sodium pentobarbital was immediately transferred to chilled glass tubes containing heparin (0.2 ml of 1000 USP/ml of solution per 10 ml of blood). The plasma was separated in a refrigerated centrifuge at 2–4°C; 3.0 ml of plasma was added to an Erlenmeyer flask containing four pieces of filter paper impregnated with about 5.0 μCi of cholesterol-26-^{14}C (specific activity 55 mCi/mmole, Amersham/Searle, Corp., Chicago, Ill.). The flask was shaken overnight in a Dubnoff shaker at 0–2°C. The radioactivity of free and esterified cholesterol was determined in an aliquot (see below), and 1 ml of the plasma was injected intravenously in certain groups of animals. The radioactivity of the injected plasma unesterified cholesterol was 0.81 ± 0.10 (mean ± SE) and 0.72 ± 0.15 μCi/ml in the resting and stimulated animals, respectively; 90% of the total radioactivity was present in the free cholesterol fraction. In other rats, about 0.5 μCi of bicarbonate-^{14}C (specific activity 4.0 mCi/mmole, Amersham/Searle, or 8.3 mCi/mmole, New England Nuclear Corp., Boston, Mass.) dissolved in saline solution was injected intravenously.

Rats were anesthetized with sodium pentobarbital administered intraperitoneally (4 mg/100 g); additional intravenous anesthesia was used as needed. A femoral vein was cannulated for blood sampling; between sampling, 2.5% glucose in saline was infused at the rate of 1.2 ml/hr. The expired CO_2 was collected starting simultaneously with the injection of radioactive tracer. The expired air was then drawn continuously through 15- or 20-ml quantities of ethanolamine–ethylene glycol monomethyl ether 1:2 (v/v) (6).

Blood samples of 0.15 ml were obtained from the femoral cannula 2, 4, 8, 15, 30, 60, 90, 120, 150, and 180 min after the injection of labeled material; 1 ml of blood was withdrawn in the last period. The samples were collected in chilled heparinized tubes and centrifuged immediately in a refrigerated centrifuge at 2–4°C. The cannula was flushed with blood before the withdrawal of samples; after sampling, the excess blood was returned to the animals as well as an additional amount of 0.15 ml of 2.5% glucose in saline.

The rats had access to food up to the time of the experiment and were assigned to four groups according to a strictly random procedure: (*a*) resting animals injected with cholesterol-26-^{14}C; (*b*) stimulated rats injected with cholesterol-26-^{14}C; (*c*) resting animals injected with bicarbonate-^{14}C; (*d*) stimulated animals injected with bicarbonate-^{14}C. Body weights in grams were, respectively, 452 ± 70 (6), 553 ± 76 (6), 578 ± 34 (4), and 460 ± 50 (4) (mean ± SE; number of animals in parentheses). In groups (*b*) and (*d*), the hind legs were stimulated with a Grass SD5 stimulator (Grass Instrument Co., Quincy Mass.) at the rate of 3/sec for 15 min before the injection of the labeled material and throughout the observation; the stimulus duration was 20 msec with a peak-to-peak amplitude of 20 ma (5).

Analytical Methods

Hematocrits in the blood samples of rats injected with labeled bicarbonate were determined by means of heparinized capillary tubes which were centrifuged for 3 min (International microcapillary centrifuge, model MB).

Total plasma cholesterol was determined in an alcohol–acetone extract of the lipoprotein preparation as well as in the blood sample obtained at 180 min (11). A similar extract was made of all plasma samples, and free cholesterol was determined in an aliquot by gas–liquid chromatography with cholesteryl propionate as an internal standard. Esterified cholesterol in the lipoprotein preparation and in the last sample of blood (180 min) was computed as the difference between total and free cholesterol; the ratio was assumed to be constant throughout the experiment. The CO_2 content in the collecting fluid through which the expired air had been drawn was analyzed by a slight modification of the Peters and Van Slyke method for CO_2 content of plasma (12).

Radioactivity was assayed in an aliquot of the alcohol–acetone extract, which was taken to dryness and dissolved in a toluene scintillator fluid containing 2,5-diphenyloxazole (PPO) (5.0 g/liter) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (M_2 POPOP) (0.1 g/liter); the counts obtained were considered to represent total cholesterol radioactivity. In another aliquot, after the addition of carrier, free cholesterol was precipitated with digitonin, washed and dried, dissolved in *p*-dioxane, and transferred quantitatively to counting vials with the toluene scintillator fluid. The radioactivity of the expired CO_2 was measured in an aliquot of the collecting fluid (13). Radioactivity assays were carried out with a Packard liquid scintillation spectrometer, model 3003; the figures reported here were computed as dpm by an automatic external standardization method (14).

Conversion of Cholesterol-26-^{14}C to Glucose-^{14}C

Two series of experiments were conducted. *Series I.* Six rats (body wt 241 ± 7 g, mean ± SE) were anesthetized with sodium pentobarbital administered intra-

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peritoneally (4 mg/100 g) and a lateral tail vein was cannulated; additional intravenous anesthesia was given as needed. Saline was infused at the rate of 1.2 ml/hr with a Harvard infusion pump. Cholesterol-26-^{14}C was prepared as reported elsewhere (5) and 10 μCi were injected intravenously. 90 min later, blood obtained from the abdominal aorta was heparinized, introduced into chilled tubes, and centrifuged in a refrigerated centrifuge; the plasma was separated and glucose was determined in an aliquot with an enzymatic method (Glucostat, Worthington Biochemical Corp., Freehold, N.J.) (15). To the remaining plasma, after dilution with water, 5% zinc sulfate and 2.2% barium hydroxide solutions were added (15), and the precipitate was removed by centrifugation. The supernatant was lyophilized overnight and the residue was dissolved in 70% ethanol. After centrifugation and concentration to 0.3 ml, the samples were chromatographed on thin-layer plates (silica gel 5762 Brinkmann,) and the following systems were used: (I) *n*-butanol–95% ethanol–water–acetic acid 50:32:17:1, (v/v); (II) ethyl acetate–acetic acid–water 3:3:1 (v/v); and (III) *n*-butanol–acetone–water 4:5:1 (v/v). A thin longitudinal strip of the plates was developed with the glucose oxidase reagent dissolved in 70% ethanol. The adjoining zone was scraped and eluted with 2 ml of 70% ethanol, and glucose was determined in an aliquot with the glucose oxidase method. To duplicate 0.5-ml aliquots 10 ml of 20% BBS-3–toluene scintillator (Beckman Corp., Anaheim, Calif.) was added, and the radioactivity was assayed.

Series II. Six rats (body wt 208 ± 4 g, mean ± SE) were injected with 5 μCi of cholesterol-26-^{14}C and studied as in series I, but in half the animals the hind legs were stimulated throughout the observation as described previously. The specific activity of glucose was determined as in series I; thin-layer chromatography was carried out with system I. The radioactivity in plasma glucose was calculated as the percentage of the injected dose based on a plasma volume of 3.5% of body weight (see below).

Computation of Turnover Rates

The more general assumptions implicit in multicompartmental analyses have been discussed in detail (16–18); specific assumptions regarding our models are summarized below.

A simplified model of the relationships between the injected radioactive cholesterol and the appearance of labeled CO_2 is shown in Fig. 1. Three pools are minimally involved: 1) cholesterol; 2) HCO_3–CO_2; and 3) expired CO_2. The vectors denote the transport of a substance into and out of a pool and also the chemical synthesis and degradation that result in the appearance

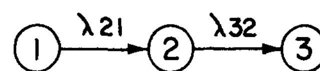

FIG. 1. A model that represents three minimally involved pools in the oxidation of the side chain of cholesterol to CO_2. *1*, cholesterol pool; *2*, HCO_3^-–CO_2 pool; *3*, expired CO_2. λ's represent fractional turnover; in the text, pool mass is represented by Q.

and disappearance of the substance in the pool. The mass of the pools is represented by Q_n in mg or mmoles/100 g of body weight. Fractional turnover, λ_{nm}, is the percentage of substance transferred per unit time from pool m to pool n and has the dimension min^{-1}. Turnover rate, k_{nm}, is the amount of substance transferred per unit time and has the dimension mg/min; it is calculated as the product of $Q_m \cdot \lambda_{nm}$.

We have assumed: 1) that the experiments have been conducted in a steady state, i.e., they have involved pools where the rates of removal of substances (cholesterol, CO_2) are considered to be equal to the rates of replacement;[1] 2) that the fractional turnover rates (λ_{nm}) were constant; 3) that the ^{14}C-labeled tracers were distributed and utilized in a manner indistinguishable from the corresponding traced substances; and 4) that the substance being studied was uniformly distributed within a given compartment during the experiment. Additional specific assumptions are discussed in Results: Multicompartmental Analysis.

Computations for the kinetic analysis were performed with the computer program SAAM-23 (19). Initial estimates for Q's and λ's were derived when appropriate from published reports or were assumed from approximations in the present studies. The fitting of data to models conformed to the outline discussed by Berman, Shahn, and Weiss (20), i.e., (*a*) choice was made of a type of model; (*b*) data fitting was carried out by an iterative process by the computer program; (*c*) judgment was made on the compatibility of the model with the data; and (*d*) the model was revised when found inconsistent with the data.

RESULTS

Hematocrit Values

The hematocrit values determined in the rats injected with bicarbonate-^{14}C were relatively constant

[1] This assumption was clearly not true in the case of the bicarbonate pool, as noted below (see Results; rates of CO_2 expiration in the breath were not constant throughout the experiment). We have not studied the influence of a nonsteady state with respect to the bicarbonate pools upon the estimated rates of cholesterol side-chain degradation in resting and stimulated rats. However, this discrepancy should have no influence upon the conclusions which are eventually reached in the present study. A more thorough analysis of cholesterol side-chain oxidation to CO_2 would require additional data and careful consideration of the complications which arise from variable rates of CO_2 excretion.

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throughout the experiments, with no significant differences between the resting and the stimulated animals (45 ± 0.6%, mean ± SE, n = 8).

Plasma Unesterified Cholesterol

The levels of plasma free cholesterol in the rats injected with labeled lipoproteins are shown in Table 1. The initial and final values were not significantly different in resting and stimulated animals. The stimulated rats tended to show lower levels, but the values were not consistently different from those in the resting animals.

TABLE 1 PLASMA FREE CHOLESTEROL IN RATS INJECTED WITH CHOLESTEROL-26-^{14}C-LABELED LIPOPROTEINS

	Number of Rats	Plasma Unesterified Cholesterol	
		Initial	Final
Resting	6	22.6 ± 1.3*	25.2 ± 1.1
Stimulated	6	21.0 ± 1.1	19.2 ± 2.7
P (t test)		NS	NS

* Mean ± SE.

Rates of Respiratory CO_2 Excretion

The rates of CO_2 expiration for resting and stimulated rats are shown in Table 2. The latter expired CO_2 about 2.5 times faster than the resting animals. The rates of CO_2 expiration were not steady throughout the 3-hr period of study, decreasing in the stimulated group and increasing in the resting group as the experiment progressed.

CO_2 Specific Activity and Rate of Excretion of $^{14}CO_2$ after the Injection of Cholesterol-26-^{14}C and Bicarbonate-^{14}C

CO_2 specific activities after the injection of labeled cholesterol (Fig. 2, left) were not significantly affected by repeated muscular contraction even though the rates of $^{14}CO_2$ excretion as reported previously (5–7) increased markedly in the stimulated animals (Fig. 2, right). After the injection of bicarbonate-^{14}C, CO_2 specific activities were lower in the stimulated animals (Fig. 3, left); as the experiment progressed, the bicarbonate pool was turned over much more rapidly in the stimulated group, as evidenced by the more rapid fall in CO_2 specific activity. The rates of increase of $^{14}CO_2$ excretion were much greater in the stimulated group than in the resting controls (Fig. 3, right).

Plasma Cholesterol-^{14}C Specific Activity

The specific activities of plasma esterified and free cholesterol are shown in Fig. 4. The specific activity of the esterified cholesterol was lower in the resting than in the stimulated rats; the specific activity of plasma free cholesterol was similar in resting and in stimulated rats. It decreased very rapidly; a 50% fall occurred within 5 min of the injection of labeled lipoproteins. No such rapid decline was observed in the specific activity of esterified cholesterol.

Multicompartmental Analysis

Usually, the rate of irreversible disposal of a circulating metabolite can be calculated by measuring the specific activity of the substance in plasma (21, 22). The present experiment was too brief to permit us to calculate the irreversible disposal rates from the measurements of the specific activity of plasma cholesterol; days or weeks rather than hours are necessary to carry out such an analysis (23). However, if the side chain of cholesterol is oxidized directly to CO_2 and if there is no alternate metabolic pathway for its degradation, the rate of irreversible cholesterol disposal can be approximated by a combined analysis of cholesterol-^{14}C and $^{14}CO_2$ data as shown by Myant and Lewis (2). Based on these assumptions, our analysis was arbitrarily limited to the free cholesterol fraction and was performed as follows. First, the plasma cholesterol-^{14}C and $^{14}CO_2$ data were converted to percentages of the injected ^{14}C in the plasma free cholesterol compartment and in breath CO_2 (a terminal compartment). The exchange and irreversible disposal of free cholesterol could then be described by a two-compartment model. The body bicarbonate–CO_2 compartments could be described by a four-compartment model, including breath CO_2 as the fourth compartment, as reported earlier by Shipley et al. (24). Then the cholesterol and bicarbonate models could be coupled to form a six-compartment model of the oxidation of the side chain to CO_2. All fractional rate con-

TABLE 2 RATES OF CO_2 EXPIRATION IN RATS

	$^{k}CO_2$ (mmoles/min)					
	Cholesterol-26-^{14}C-injected Rats		P (t test)	Bicarbonate-^{14}C-injected Rats		P (t test)
	Initial	Final		Initial	Final	
Resting	0.18 ± 0.01 (6)†	0.23 ± 0.01 (6)	<0.02	0.14 ± 0.02 (4)	0.27 ± 0.01 (4)	<0.001
Stimulated	0.56 ± 0.05 (6)	0.46 ± 0.03 (6)	NS	0.54 ± 0.02 (4)	0.32 ± 0.01 (4)	<0.01

* Calculated from a 30-min interval.
† Mean ± SE. Number of animals in parentheses.

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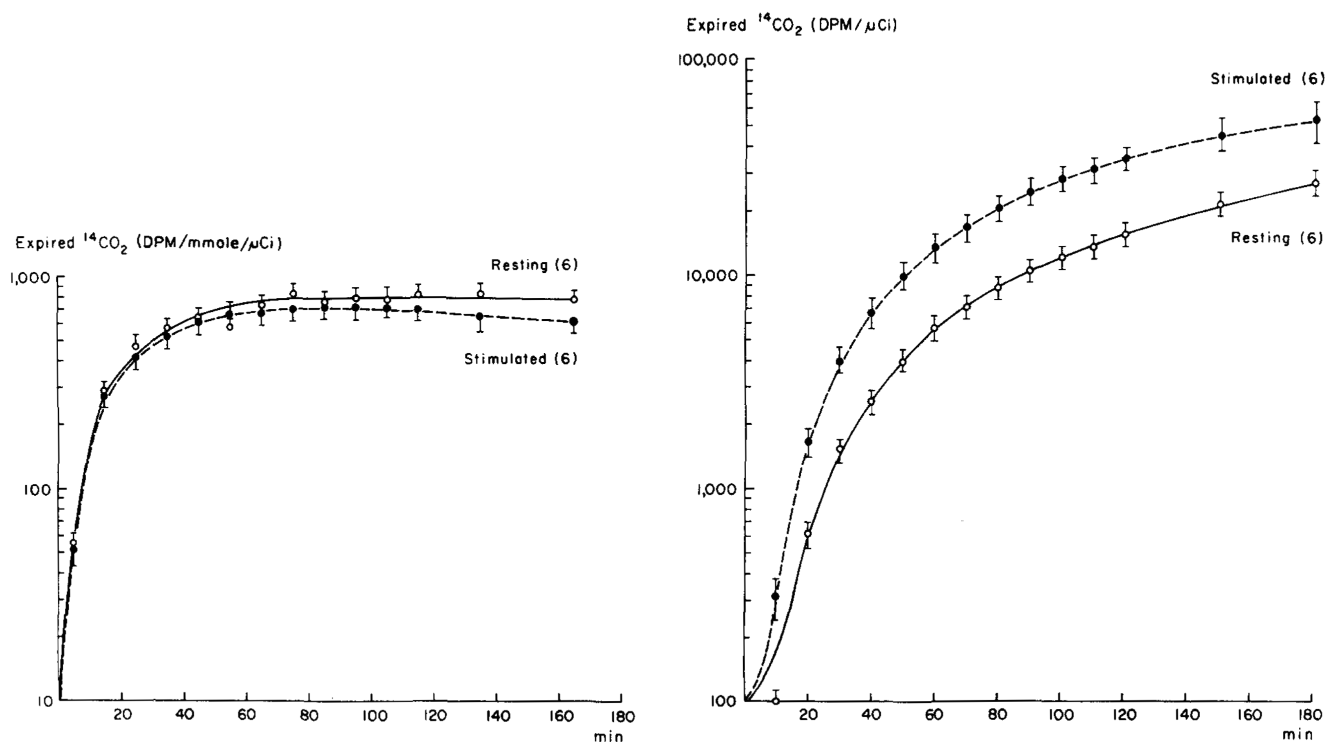

Fig. 2. Excretion of $^{14}CO_2$ in rats injected with cholesterol-26-^{14}C-labeled lipoproteins at 0 time. Left, CO_2 specific activity; right, $^{14}CO_2$ cumulative excretion. In this and the following figures, each point indicates the average for the group ±SE. Excretion is represented at mid-point for the interval; cumulative excretion is represented at the end of the interval and is standardized for 1 μCi of ^{14}C injected. Number of animals is between parentheses.

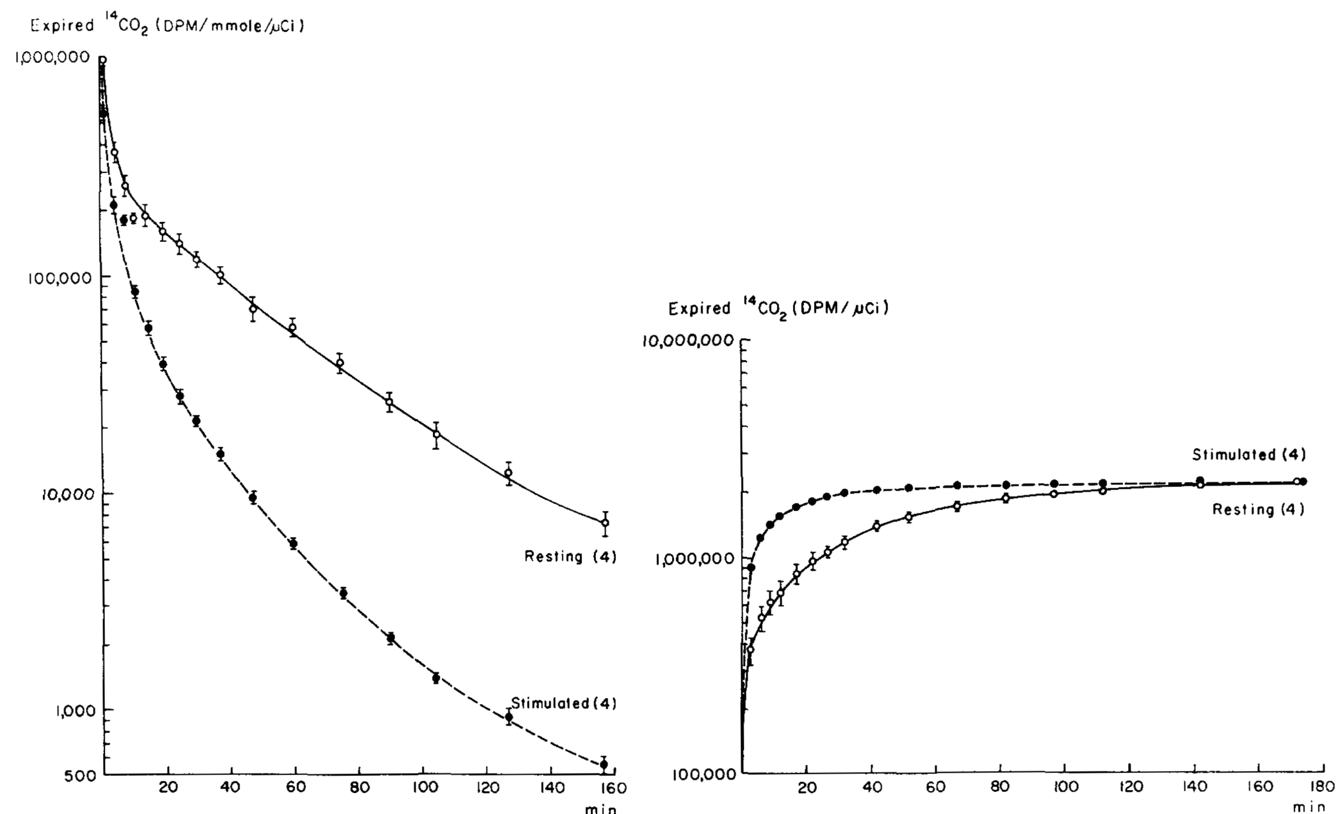

Fig. 3. Excretion of CO_2 in rats injected with bicarbonate-^{14}C at 0 time. Left, CO_2 specific activity; right, $^{14}CO_2$ cumulative excretion. SE not shown were too small to be drawn. The 3-hr excretion has been considered to represent 100% of the injected ^{14}C. Symbols, see Fig. 2.

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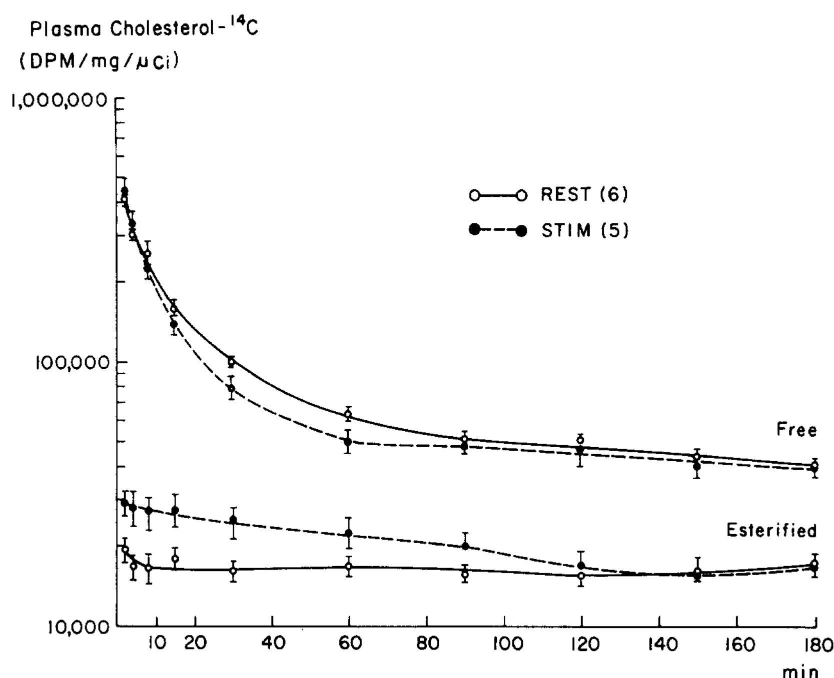

FIG. 4. Plasma specific activity of free and esterified cholesterol in rats injected with cholesterol-26-^{14}C-labeled lipoproteins at 0 time. Symbols, see Fig. 2.

stants that define the exchange of either the two cholesterol or four bicarbonate compartments were determined by computer analysis (19). The rate of irreversible disposal of cholesterol, which we assumed would approximate the rate of degradation of the cholesterol side chain, was the only unknown parameter; moreover, initial estimates for this rate could be approximated from values reported in the literature for bile acid production and adrenal steroid synthesis. Using the respired $^{14}CO_2$ and plasma cholesterol-^{14}C data, we then attempted to determine this rate of side-chain oxidation.

Free Cholesterol Exchange and Irreversible Disposal

Two approaches were taken in the analysis of the plasma free cholesterol-^{14}C data. One approach was to analyze separately the data obtained from each individual animal. A second approach was to take information derived from the first analysis (e.g., Q_2) and then reanalyze all of the composite data in a single analysis which would serve as the basis for subsequent analysis of the coupled free cholesterol and bicarbonate–CO_2 compartments. In each case, a two-compartment model having four λ's was used (Fig. 5) and the object of the analysis was to estimate λ_{01}, λ_{12}, and λ_{21}. Compartment 1 represents the free cholesterol of the plasma; compartment 2 represents the apparent exchangeable mass, which includes free cholesterol in the liver, the adrenal glands, and the red blood cells; λ_{01} indicates the exchange of plasma cholesterol with the cholesterol of the rest of the body;[2] λ_{02} represents the irreversible disposal of cholesterol through bile acid excretion and adrenal steroid formation. The size of pool 1 was taken as 0.735 mg/100 g of body weight, based upon the levels of free cholesterol observed and an assumed plasma volume of 3.5% of body weight (25). The mass of compartment 2 was assumed initially to be 9 mg/100 g of body weight in both groups of animals; it mainly represents free cholesterol in the liver (8 mg/100 g) (26). However, it also included adrenal cholesterol (0.3 mg/100 g; adrenal weight in our male rats equals 7 mg/100 g of body weight, with a total cholesterol content of 4.45 mg/100 mg wet weight [27]; no attempt was made to differentiate the free and esterified fractions, since their contribution to Q_2 is small compared with the liver). Finally, compartment 2 also included red blood cell free cholesterol (0.7 mg/100 g, assuming a concentration equal to that in the plasma) (28). λ_{02} (k_{02}/Q_2) was derived from a value of k_{02} estimated in the resting rats as 3.3 mg/100 g/day from reported biliary excretion (29) and adrenal steroid production of 0.6 mg/100 g/day (30). The biliary excretion seems a likely estimate, since other

[2] No recycling of cholesterol is shown since the exchangeable pool is so large that it probably returns a negligible quantity of ^{14}C into compartment 1 during the experimental period. As the analysis progressed, it was apparent that exchange with the third compartment did influence the terminal shape of the curve. However, the model was not modified in this respect since it did not affect the rest of our calculations.

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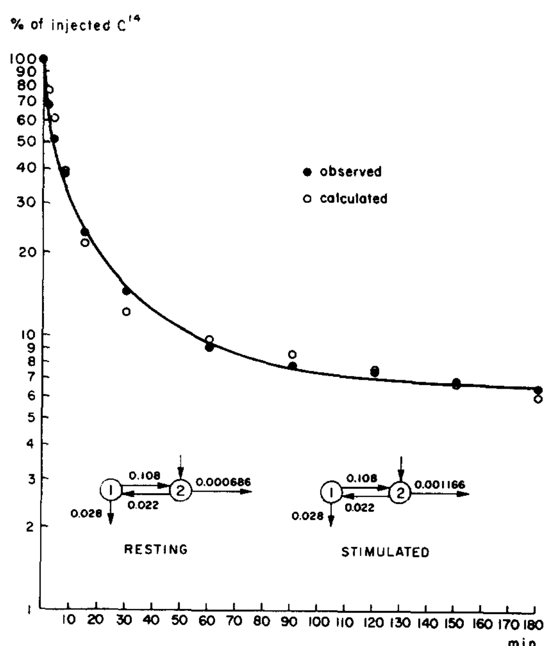

FIG. 5. Disappearance of radioactive free cholesterol in the plasma of rats injected with cholesterol-26-^{14}C-labeled lipoproteins. The observed curve represents the combined average of six resting and five stimulated animals (see text). The cholesterol specific activity values for the individual groups are shown in Fig. 4. The least squares fit has been calculated with the fractional rate constants indicated in the model for the resting group. A similar fit was obtained when the parameters for the stimulated group were used. As noted in the text, the models were defined by information derived from steady-state considerations and the literature, as well as from the data shown. Thus, Q_1 was measured experimentally and k_{02} was estimated from literature values, isotopic data, and steady-state considerations (see text). Fractional standard deviations for the several parameters which were determined solely from the isotopic data by the computer analysis were as follows: λ_{01}, ± 0.15; λ_{21}, ± 0.09; and λ_{12}, ± 0.17.

workers have reported values between 1.0 and 4.5 mg/100 g/day (31, 32); the adrenal steroid production is probably a magnified value obtained under maximal ACTH release (33). λ_{02} for the stimulated animals was estimated as 1.7 times the resting values, from demonstrated increases in bile acid synthesis in another series of rats.[3]

In the first approach, data obtained for individual animals were analyzed, and λ_{01}, λ_{12}, and λ_{21} were calculated by the computer; the results are shown in Table 3. In this analysis, λ_{02}, which is not shown in Table 3, was fixed at 0.00026 min^{-1} (resting) and 0.00044 min^{-1} (stimulated). However, after the analysis had been completed, a new estimate for this parameter was necessary, since Q_2 based on the mean λ's of resting and stimulated was found to be 3.6 mg instead of the originally assumed 9 mg. That is, computer data analysis indicated that only a portion of the liver, adrenal, and red blood cell free cholesterol had mixed rapidly with plasma free cholesterol. New estimates of λ_{02} were then made with the same values for k_{02} as indicated above; these values for λ_{02} were used in the second analysis of the composite data (Fig. 5). The new values for λ_{02} do not influence the solution derived by the computer (Table 3), since the magnitude of λ_{02} is small relative to all the other λ's.

TABLE 3 FRACTIONAL RATE CONSTANTS OF FREE CHOLESTEROL IN INDIVIDUAL ANIMALS

Rat No.	Fractional Rate Constants (min^{-1})		
	λ_{12}	λ_{21}	λ_{01}
R_1*	0.022	0.066	0.022
R_2	0.026	0.118	0.023
R_3	0.028	0.119	0.027
R_4	0.019	0.103	0.029
R_5	0.039	0.170	0.034
R_6	0.030	0.122	0.023
Mean ± SD	0.027 ± 0.007	0.116 ± 0.034	0.026 ± 0.005
S_1	0.020	0.128	0.049
S_2	0.016	0.069	0.020
S_3	0.014	0.080	0.017
S_4	0.020	0.105	0.034
S_5	0.022	0.144	0.029
S_6	0.011	0.081	0.028
Mean ± SD	0.017 ± 0.004	0.101 ± 0.030	0.030 ± 0.011

* R, resting; S, stimulated.

In the second analysis, all of the data were combined by taking the mean values of all rats from both the resting and stimulated groups. This seemed justified by several criteria. First, inspection of the curves in Fig. 4 suggested that the resting and stimulated groups were not markedly different. Second, comparison of specific activities for resting and stimulated groups at each point in time showed only rare significant differences. Finally, the computer data analysis shown in Table 3 indicated that there was considerable overlap between groups for all parameters and that the mean values for resting plus stimulated groups were similar to the values for either individual group. The results of the second analysis are shown in Fig. 5. Close correspondence was seen between the observed disappearance of free cholesterol-^{14}C from the plasma and the values calculated from the parameters shown at the bottom of Fig. 5. As noted above, the fit, during this time period, is virtually unaffected by the two values of λ_{02} which were used for the resting and stimulated groups.

Bicarbonate Turnover

Fig. 6 shows a kinetic analysis of $^{14}CO_2$ excretion in rats injected with bicarbonate-^{14}C. As noted above, the rate of $^{14}CO_2$ excretion was faster in the stimulated rats. The model shown on the right-hand side of the figure

[3] More recent studies with bile fistula rats, in which bile from donor animals was reinfused into the small intestine, indicate that the increase in bile acid synthesis associated with muscular stimulation is larger in animals without reinfused bile. (M. R. Malinow, P. McLaughlin, and I. Pierovich. Unpublished data.)

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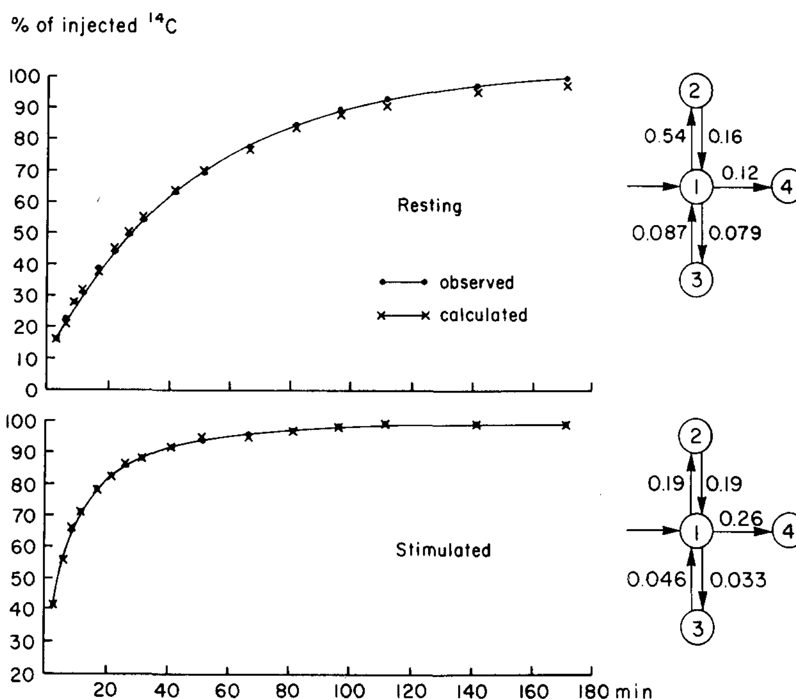

FIG. 6. Average cumulative excretion of $^{14}CO_2$ in rats injected with bicarbonate-^{14}C. Compartments 1, 2, and 3 correspond approximately to extracellular bicarbonate, intracellular bicarbonate, and an indeterminate pool, respectively; compartment 4 represents expired CO_2. The least squares fit has been calculated with the fractional rate constants indicated in the models.

is similar to the bicarbonate pool system described in rats by Shipley et al. (24). The reader is referred to the paper of Shipley et al. for definition of the compartments, the exact nature of which need not be elaborated here since it is not essential for the present analysis (18). Values for the fractional rate constants derived by the SAAM-23 program are represented in the figure. The computer curves calculated from the values for the fractional rate constants are also shown. A close correspondence between the calculated and the observed values was obtained. The present analysis suggests that CO_2 flow from the extracellular to the intracellular compartments is apparently reduced during muscular stimulation and that at the same time the fractional rate of excretion from the extracellular fluid into the breath is increased. However, this analysis tends to be more accurate during the earlier than during the later stages of the experiment, when the rates of CO_2 excretion were varying. As noted above, the influence of varying rates of CO_2 expiration upon the analysis was not studied, but this consideration should have little influence upon the final conclusions that we have reached. For example, data analysis could be restricted to the first 90 min (during which time the rates of CO_2 excretion were approximately constant) without influencing significantly any of the subsequent calculations.

Formation of $^{14}CO_2$ Derived from the Side Chain of the Cholesterol Molecule

The analysis so far has described the cholesterol and the bicarbonate systems separately. In order to represent the oxidation of the side chain of cholesterol to CO_2, we must now connect them as indicated in the lower part of Fig. 7. All fractional rate constants have already been determined except λ_{32}, which, as mentioned above, is estimated from reported values of bile acid excretion and adrenal steroid production rates (29, 30).

A comparison between the computer-predicted and the observed $^{14}CO_2$ excretion curves with these values is shown in Fig. 7. The calculated curve is the zeroth iteration (19) and depends on the assumed value for λ_{32}. If all λ's except λ_{32} are held constant, the computer analysis should be able to vary λ_{32} until a best fit for the $^{14}CO_2$ data is obtained. However, we did not proceed further with the computer analysis, since obviously a fit to the data could be obtained only by reducing markedly the value of λ_{32} to the extent that k_{32} $(Q_2 \cdot \lambda_{32})$ would not be compatible with the estimated side-chain oxidation based upon reported bile acid and adrenal steroid secretion rates. Accordingly, we considered the alternate possibility that the assumed values for k_{32} and λ_{32} were within the correct order of magnitude but

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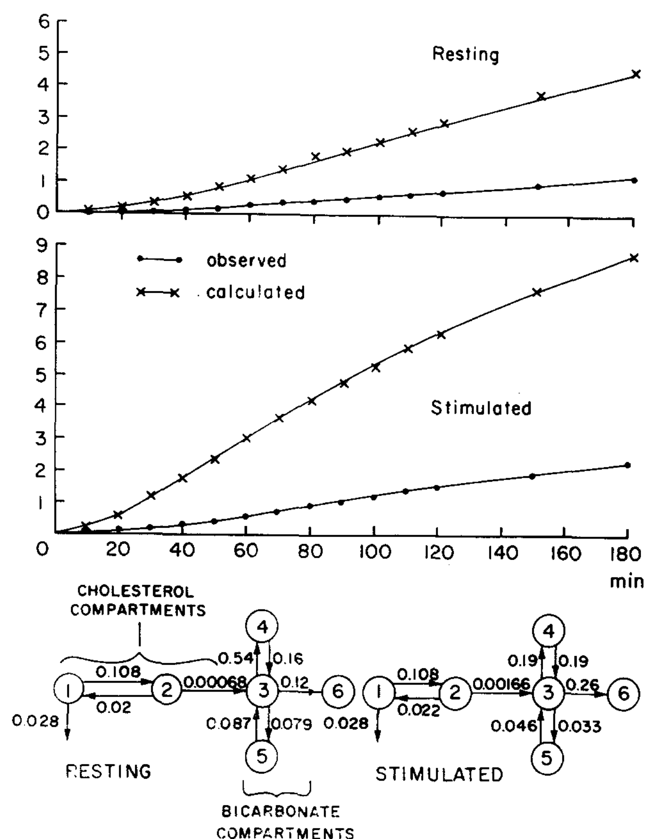

Fig. 7. Average cumulative excretion of $^{14}CO_2$ in rats injected with cholesterol-26-^{14}C-labeled lipoproteins. The calculated curve is the zeroth iteration obtained by computer analysis from the fractional rate constants indicated in the models.

that the model was incorrect, especially in our assumption that the side chain of cholesterol is oxidized directly to CO_2.

Glucose as an Intermediate in Cholesterol Side-chain Oxidation

Assuming that propionyl CoA is formed in the liver from the side chain of cholesterol during the reactions that lead to the synthesis of bile acids (34), one might expect that some of the propionyl CoA would be converted to glucose, which is known to be formed readily from propionate (35); furthermore, gluconeogenesis is one of the major functions of the liver. Thus, it seemed reasonable to us that most of the propionyl CoA which formed in the liver was not oxidized to CO_2 in the liver but was first converted to glucose, which was then oxidized to CO_2 primarily by the extrahepatic tissues. Consequently, an expanded model, shown on the left-hand side of Fig. 8, was formulated. Here the cholesterol and the bicarbonate pools are shown together with a set of glucose pools as reported in rats by Baker et al. (36).[4] Pool 7 represents plasma glucose; pool 8, extraplasma glucose; and pool 9, nonglucose intermediates. Flux from compartment 2, so far represented by λ_{32}, was split into three λ's; the sum of λ_{32}, λ_{72}, and λ_{02} was held constant and equal to the value of 0.000686 and thus conformed to the previous estimates (Fig. 7). As indicated in the figure, λ_{32} now represents a relatively small and direct pathway of side-chain oxidation to CO_2; λ_{72}, oxidation of the side chain by way of propionyl CoA conversion to glucose; and λ_{02}, conversion of propionyl CoA to substances that do not form appreciable $^{14}CO_2$ during the experimental period. Their sum remained fixed, but each new λ was allowed to vary until a best fit to the $^{14}CO_2$ data was obtained. The values for λ_{02}, λ_{72}, and λ_{32} were thus calculated by the computer program and a solution was arrived at that fitted the data of $^{14}CO_2$ obtained. The close correspondence of the data in the resting animals to the least squares fit is shown on the right side of Fig. 8. The analysis, therefore, suggests that only a small fraction of the side chain of cholesterol is oxidized directly to CO_2 ($\lambda = 0.000086$ min^{-1}). It is also apparent that if a fraction of the propionyl-^{14}C CoA is converted to glucose, the observed rate of excretion of $^{14}CO_2$ after the injection of cholesterol-26-^{14}C can be explained, in part, by the oxidation of glucose-^{14}C.

In order to obtain direct confirmation of the assumption that glucose is an intermediate in the oxidation of the cholesterol side chain, we constructed by computer analysis (19) a theoretical curve for the specific activity of plasma glucose (compartment 7, Fig. 8) after the injection of cholesterol-26-^{14}C. The theoretical curve for plasma glucose-^{14}C reached a maximum value at 90 min. Subsequently, two series of rats were injected intravenously with cholesterol-26-^{14}C, and plasma glucose-^{14}C was measured at 90 min. Table 4 shows that glucose separated from the plasma of rats (series I) was radioactive and that the specific activity was similar in three different chromatographic systems. Table 5 shows that the specific activity of glucose was probably similar in both the resting and the stimulated rats of series II. In all animals, the amount of radioactive glucose was within the order of magnitude predicted by the computer analysis; thus, the hypothesis

[4] Differences between the experimental procedure of Baker et al. (36) and that of the present study will probably alter the rate constants in our animals; therefore, the analysis should be accepted only as a first approximation.

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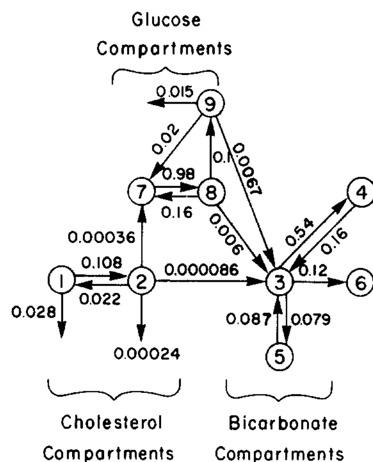

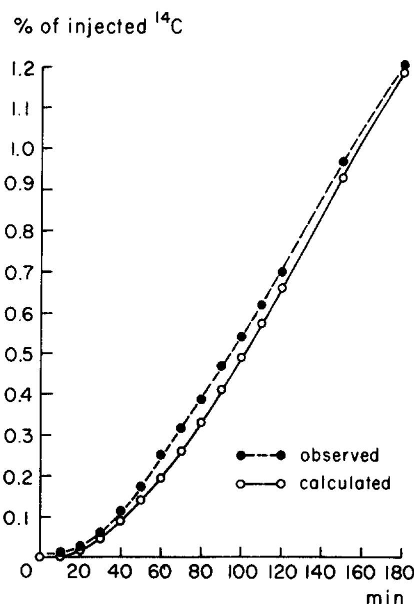

FIG. 8. Average cumulative excretion of $^{14}CO_2$ in resting rats injected with cholesterol-26-^{14}C-labeled lipoproteins: effect of inclusion of alternate pathways of degradation, such as conversion of the cholesterol side chain to glucose prior to formation of $^{14}CO_2$. Note that compartment 9 is a nonglucose compartment which is derived, in part, from glucose and which also recycles carbon to glucose (36). The curve shows the same data as in the upper part of Fig. 7, drawn to a different scale. The least squares fit has been calculated with the fractional rate constants indicated in the model.

TABLE 4 Specific Activity of Glucose 90 min after the Injection of Cholesterol-26-^{14}C in Rats

	Number of Rats	Plasma Glucose Specific Activity
System I	6	260 ± 30*
System II	6	240 ± 36
System III	5	272 ± 44

Animals were injected at 0 time with 10 μCi of labeled cholesterol. Glucose-^{14}C was isolated by thin-layer chromatography with three separation systems: (I) *n*-butanol–95% ethanol–water–acetic acid 50:32:17:1 (v/v); (II) ethyl acetate–acetic acid–water 3:3:1 (v/v); and (III) *n*-butanol–acetone–water 4:5:1 (v/v).

* Mean ± SE.

TABLE 5 Specific Activity of Glucose 90 min after the Injection of Cholesterol-26-^{14}C in Rats

	Number of Rats	Plasma Glucose Specific Activity		Glucose-^{14}C in Plasma Compartment	
		Mean	Range	Observed (mean)	Expected*
		dpm/mg		*% of injected ^{14}C*	
Resting	3	280	250–320	0.038	0.070
Stimulated	3	380	250–520	0.043	

Animals were injected at 0 time with 5 μCi of labeled cholesterol. Glucose was isolated by thin-layer chromatography with system I (see Table 4).

* See text.

that glucose is an important intermediate in the metabolism of the side chain of cholesterol was substantiated.[5]

DISCUSSION

The effects of repeated muscular contractions on the degradation of cholesterol are promptly detected in bile fistula animals; in rats injected with ring-labeled cholesterol, differences in the excretion of radioactive bile acids between resting and stimulated animals can be detected as early as 30 min after the induction of muscular contractions (37). It seems theoretically possible that other noninvasive methods more easily applicable to man would detect acute changes in cholesterol metabolism after the injection of suitably labeled cholesterol, e.g., by comparing the specific activities of plasma free cholesterol and expired $^{14}CO_2$ to estimate

[5] All estimates of fractional rates of direct vs. indirect oxidation of the cholesterol side chain to CO_2 are highly dependent upon the assumptions we have made, especially the assumed rate of cholesterol degradation. In order to obtain a quantitative estimate of the rate of cholesterol side-chain conversion to glucose, additional experimental information is required. The turnover of glucose, the rate of cholesterol-26-^{14}C appearance in glucose, in CO_2, and in other intermediates, as well as the irreversible disposal of cholesterol should be measured in one population of animals in both resting and stimulated states. Such information is not presently available.

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rates of cholesterol side-chain oxidation (2). We have attempted to use such an approach to study the acute effects of muscular stimulation upon cholesterol degradation in rats.

The study of the specific activity of plasma cholesterol–time curves after the injection of labeled cholesterol has been the subject of numerous reports. Such studies early demonstrated that the half-life of cholesterol varied between one and several weeks depending upon the species of animals under consideration (38) and that the plasma specific activity of the free and esterified fractions tended to equalize in 24–48 hr (39). Accordingly, most authors have disregarded the early part of the curve, and the distinction between the metabolism of free and esterified cholesterol was difficult to establish after equilibration of these fractions. In order to study separately the metabolism of free and esterified cholesterol, measurements must be carried out shortly after the injection of the tracer. Our observations demonstrate that the initial rate of removal of free cholesterol from plasma is much faster than that of esterified cholesterol as reported by Nestel, Havel, and Bezman (40) after the injection of chylomicrons in dogs. Such a differential rate of removal in our lipoprotein preparation may indicate that, unlike foreign material, the labeled lipoproteins were not removed *in toto* in any significant amount and that they behave in a "physiological" manner. Such a possibility is obviously important in experiments that involve the intravenous injection of cholesterol (37). The present analysis also shows that about 10% of the plasma free cholesterol is replaced each minute in the rat. This value corresponds to an exchange of plasma free cholesterol of 120 mg/100 g/24 hr, or to a daily exchange equivalent to 150 times the content in plasma of rats. Such a fast exchange rate in the resting animals would prevent us from detecting in the plasma any increase in the irreversible disposal rate associated with muscular stimulation (7) and might explain the similarity between the curves of the specific activity of plasma free cholesterol in resting and stimulated animals. Assuming that the same rapid fractional turnover takes place in man, 400 g of free cholesterol would be exchanged from the plasma each day! This fast turnover has been emphasized previously (41), based on the observation of Eckles et al. (42), who reported an equilibration half-time of 0.3 hr between the free cholesterol of plasma and liver in dogs.

In the present study, the relationship between the specific activity of free cholesterol in the plasma and the liver was not determined directly. Our analysis suggests, however, that only $^1/_2$ to $^1/_3$ of the free cholesterol in the liver was involved in the early exchange; thus, it only partially agrees with the results of Eckles et al. (42). However, our observations closely concur with those of Sodhi and Kalant (43), who suggested that in the liver of normal rats there are at least two distinct free cholesterol compartments of different specific activities. In the present analysis, we hypothesized that only the hepatic free cholesterol pool which equilibrates rapidly with plasma free cholesterol is directly involved in the degradation of cholesterol to bile acids and CO_2. The rapid appearance of labeled bile acids after the injection of ring-labeled cholesterol in resting and stimulated rats (37) is consistent with this interpretation.

The major aim of the present study was to calculate the rate of cholesterol side-chain degradation to CO_2 in resting and electrically stimulated rats during an acute observation. To our knowledge, no one has reported such an analysis. However, Myant and Lewis (2) established a rather close correspondence between the rates of cholesterol degradation in man as measured by two techniques, fecal excretion of ^{14}C-labeled bile acids and $^{14}CO_2$ excretion in the breath after injection of cholesterol-4,26-^{14}C. Moreover, a significant and positive correlation between $^{14}CO_2$ and ^{3}H-labeled bile acids was also present in resting and electrically stimulated rats injected with cholesterol-1,2α-^{3}H/26-^{14}C (7). Therefore, it seemed reasonable to expect that the data obtained in the present study would make it possible to estimate the desired rates by multicompartmental analyses as has been done for other ^{14}C-labeled metabolites (18). However, it became apparent almost immediately that the model upon which our analysis was based—a model very similar to that which is implied in the calculations of Myant and Lewis (2)—would yield estimates for rates of cholesterol side-chain degradation incompatible with the known rates of bile acid excretion in rats.

In analyzing our inability to calculate the rate of cholesterol degradation, we considered the possibility that the side chain of cholesterol is not oxidized directly to CO_2 but through some other metabolites, as was suggested earlier by Myant and Lewis (2) and by Malinow et al. (7). Other workers have published data that would make this hypothesis highly plausible. For example, Van Bruggen, Hutchens, and West (44) and Kritchevsky, Kirk, and Biggs (45) reported that fatty acids became relatively highly labeled, whereas liver glycogen became only slightly radioactive after the administration of cholesterol-U-^{14}C to rats. However, because of the design of the earlier experiments, the finding of small amounts of radioactivity in glycogen does not necessarily indicate that carbohydrate is an unimportant intermediate in cholesterol side-chain oxidation. Since propionate is probably formed in the liver during the conversion of cholesterol to bile acids (34) and is readily converted to glucose (35), we hypothesized

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that glucose is an important intermediate in cholesterol side-chain oxidation to CO_2. Our hypothesis received direct confirmation when glucose-^{14}C was detected within an order of magnitude of the predicted levels in the plasma of rats injected with cholesterol-26-^{14}C. Our model also predicts that the side chain of cholesterol is incorporated into other metabolites, which presumably would include fatty acids (44, 45); however, these alternate pathways have not been further explored in the present analysis. Myant and Lewis's (2) calculations did not take into consideration the incorporation of the side chain of cholesterol into glucose and other intermediates; the approximate correspondence of their estimates for cholesterol degradation from CO_2 data and from bile acid formation is due, at least partly, to the longer intervals of their observations.[6]

If glucose is an important intermediate in the oxidation of the side chain of cholesterol, then early changes in the degradation of cholesterol, such as those that might be effected by short-term muscular stimulation, might be detected by a study of glucose turnover and glucose formation from cholesterol under similar conditions. Moreover, since the adrenal glands play a significant role in the oxidation of cholesterol to CO_2 (6, 7), one must establish whether isocaproaldehyde originating from the side chain of cholesterol in the adrenal cortex (46) is oxidized directly to CO_2, or whether isocaproaldehyde is first converted to glucose or some other substance before it is oxidized to CO_2. Such information is not yet available and further experiments in this direction are indicated. Finally, since the endocrine system has an important bearing on the control of gluconeogenesis, studies on the significance of glucose as an intermediate in cholesterol degradation may provide clues to understanding the alterations of cholesterol metabolism observed in disturbances of the endocrine balance.

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[6] Baker, N., and H. Rostami. Unpublished data.

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