Absolute rates of cholesterol synthesis in extrahepatic tissues measured with ^{3}H-labeled water and ^{14}C-labeled substrates

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Abstract This study was undertaken to develop techniques for measuring absolute rates of sterol synthesis in extrahepatic tissues in vitro and to estimate the magnitude of the errors inherent in the use of various ^{14}C-labeled substrates for such measurements. Initial studies showed that significant errors were introduced when rates of synthesis were estimated using [^{3}H]water since about 20 nmol of water were bound to each mg of tissue cholesterol isolated as the digitonide. This source of error could be eliminated by subtracting apparent incorporation rates obtained at 0°C from those obtained at 37°C or by regenerating and drying the free sterol. In a second set of experiments, the H/C incorporation ratio in cholesterol was determined in the liver by measuring the absolute rates of hydrogen and acetyl CoA flux into sterols. The ratio of 0.69 ± 0.03 was found to be independent of the rate of hepatic cholesterol synthesis, the rate of hepatic acetyl CoA generation, or the source of the acetyl CoA. In a third set of studies, rates of incorporation of [^{3}H]water or ^{14}C-labeled acetate, octanoate, and glucose into digitonin-precipitable sterols were simultaneously measured in nine different extrahepatic tissues. Assuming that the H/C ratio measured in the liver also applied to these tissues, the [^{3}H]water incorporation rates were multiplied by the reciprocal of the H/C ratio to give the absolute rates of sterol synthesis in each tissue. When these were compared to the incorporation rates determined with the ^{14}C-labeled substrates the magnitude of the errors in the rates of sterol synthesis obtained with these substrates in each tissue could be assessed. Only [^{14}C]octanoate gave synthesis rates approaching 100% of those obtained with [^{3}H]water and this occurred only in the intestine and kidney; in the other extrahepatic tissues this substrate gave rates of 6–66% of the absolute rates. Rates of [^{14}C]acetate incorporation in sterols varied from 4 to 62% of the [^{3}H]water incorporation rates while those obtained with [^{14}C]glucose were only 2–88% of the true rates. These studies document the large and highly variable errors inherent in estimating rates of sterol synthesis in extrahepatic tissues using ^{14}C-labeled substrates under in vitro conditions.—**Andersen, J. M., and J. M. Dietschy.** Absolute rates of cholesterol synthesis in extrahepatic tissues measured with ^{3}H-labeled water and ^{14}C-labeled substrates. *J. Lipid Res.* 1979.20: 740–752.

Supplementary key words liver cholesterol synthesis · H/C incorporation ratio · glucose · acetate · octanoate

While rates of cholesterol synthesis in liver and in many extrahepatic tissues have been reported from this laboratory (1–4) as well as from many other laboratories (5–8), there is still considerable uncertainty as to the validity of many of these rate constants. When a ^{14}C-labeled substrate is utilized in a whole cell prepartion there is always the possibility that the rate of incorporation of that substance into cholesterol underestimates the absolute rate of sterol synthesis either because the rate of entry of the substrate into the cell or its metabolism to acetyl CoA is rate limiting or because the specific activity of the substrate (or that of the acetyl CoA generated from the substrate) has been diluted within the cell. To a degree, these problems can be circumvented by measuring the activity of the rate-limiting enzyme in the cholesterol biosynthetic pathway, i.e., 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase) under V_{max} conditions. However, there are also other problems associated with such assays. For example, the conditions for measuring microsomal HMG CoA reductase have not been worked out in many extrahepatic tissues, and in some of these tissues this enzymatic activity appears to be associated with subcellular particles other than microsomes (9). Furthermore, recovery of microsomes from many tissues is incomplete, so estimates of cholesterol synthesis rates expressed per unit weight of tissue may be very inaccurate. Finally, it has also been shown recently that even in the liver the activity of HMG CoA reductase can be altered as much as 4- to 8-fold simply by varying the conditions under which the microsomes are prepared (10, 11). Thus, for all these reasons, it is apparent that currently there is no method that gives absolute rates of cholesterol synthesis

Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; DPS, digitonin-precipitable sterols; APP, 4-aminopyrazolo[3,4-*d*]pyrimidine.

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from ^{14}C-labeled substrates in different tissues under in vitro conditions.

The present studies were undertaken to develop such methods and are based on the following scheme. Rates of incorporation of [^{3}H]water and ^{14}C-labeled acetate, octanoate, and glucose into digitonin-precipitable sterols (DPS) were simultaneously measured in tissue slices from ten different organs. The theoretical rates of acetyl CoA incorporation into sterols were calculated for each tissue by multiplying the rates of [^{3}H]water incorporation into DPS by the experimentally determined value of the C/H incorporation ratio in the cholesterol molecule. The theoretical rates thus obtained could be compared to the actual rates of acetyl CoA units incorporated into DPS from each of the ^{14}C-labeled substrates in order to estimate the magnitude of the error present in each tissue when these different substrates were utilized for measuring rates of sterol synthesis. In order to make these measurements, however, three different groups of experiments were necessary. The first set of investigations delineated certain technical problems that were encountered when rates of [^{3}H]water incorporation into DPS were measured. The absolute rates of hydrogen and carbon incorporation into cholesterol by the liver were measured in a second group of studies under different physiological circumstances in order to establish a valid value for the H/C incorporation ratio. In a third group of studies, this value was used to determine absolute rates of acetyl CoA incorporation into sterols in different tissues so that comparisons could be made with rates of synthesis measured with the various ^{14}C-labeled substrates. These studies confirm that rates of sterol synthesis in many extrahepatic tissues have been grossly underestimated in previously reported studies.

MATERIALS AND METHODS

Animal preparations

The female, Sprague-Dawley-derived rats used in these studies were purchased in the weight range of 150–180 g (Charles River Breeding Laboratories, Wilmington, MA) and subjected to light cycling for 2–3 weeks before use (12). During this time they were allowed free access to water and Formulab Rat Chow Diet (Ralston Purina Co., St. Louis, MO). Most studies were carried out using fed animals killed at the mid-dark phase of the light cycle but, in specific experiments, animals also were killed at the mid-light phase of the cycle or after being fasted 24 hr, fed 2% (w/w) cholestyramine for 7 days, or treated with 4-amino-pyrazolo[3,4-*d*]pyrimidine (APP) for 4 days to lower the plasma cholesterol levels (4, 12).

Tissue incubation

The animals were killed and the livers and other tissues were immediately removed, chilled, and sliced as previously described (3, 12). Various quantities of slices were incubated in 25-ml centerwell flasks containing 2.0 ml of Krebs bicarbonate buffer gassed with 95% O_2–5% CO_2. The flasks were incubated for 1.5 hr at 0°C or 37°C in a metabolic incubator shaken at 120 oscillations/min. In specific experiments Na acetate, Na octanoate, or glucose was present in the buffer and, in addition, either [^{3}H]water (10 mCi), [1-^{14}C]acetate (8 μCi), [1-^{14}C]octanoate (8 μCi), or [U-^{14}C]glucose (8 μCi) was added to the incubation medium. The amount of each of these substrates used in specific experiments is given in detail in the legends to the tables and figures.

Chemical procedures

After completion of the incubations, the CO_2 was collected from those flasks containing ^{14}C-labeled substrates as previously described (13, 14). Six ml of alcoholic KOH was then added to the flasks and they were incubated on a steam bath for 2 hr to saponify the lipids (14). The contents of the incubation flasks were then quantitatively transferred to 250-ml flasks; the sterols were extracted and precipitated as the digitonides; the precipitates were washed twice with acetone and once with diethyl ether; and the radioactivity in the digitonin-precipitable sterols was quantitated (13, 14). In one group of experiments the digitonides were split with pyridine and the free sterols were extracted into diethyl ether (15). In our hands this procedure leads to more than 98% recovery of authentic [^{3}H]cholesterol. Portions of this extract were subjected to thin-layer chromatographic analysis (16). Tissue cholesterol content also was measured colorimetrically after saponification of the tissue samples in alcoholic KOH (14).

Calculations

Several types of calculations were carried out in order to correct for differential losses in radioactivity during the synthesis of DPS from the various precursors and to normalize the incorporation rates to the same base. In tissues incubated with [^{3}H]water the total cpm in DPS was divided by the specific activity of the water in the incubation flask, by the weight of the tissue incubated (in g), and by the duration of incubation (in hr) to give synthesis rates in terms of the nmol of [^{3}H]water incorporated into DPS per g per hr ($nmol \cdot g^{-1} \cdot hr^{-1}$). Similarly, the amount of ^{14}C-labeled

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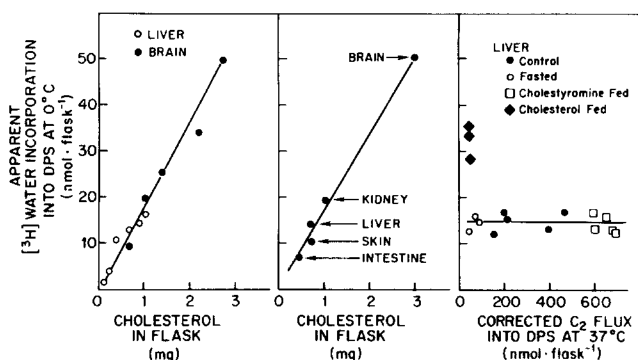

Fig. 1. Apparent [^{3}H]water incorporation rates into DPS at 0°C as a function of the amount or type of tissue in the incubation flasks or the rate of cholesterol synthesis in the tissue. Different amounts of tissue slices were incubated for 1.5 hr at 0°C in flasks containing 2.0 ml of Krebs bircarbonate buffer, 16 μmol of Na acetate, and 10 mCi of [^{3}H]water and shaken at 120 oscillations per min. In the left panel, varying amounts of either liver or brain slices were added to the flasks while, in the middle panel, 300 mg of different tissues were utilized. The amount of tissue is expressed as the amount of cholesterol present in the slices added to each flask. In the right panel, 300-mg aliquots of liver slices from each animal were incubated with [^{3}H]water at 0°C and with [1-^{14}C]octanoate at 37°C. In this latter experiment livers were obtained from control animals at different points in the light cycle or from animals that were fasted or fed cholesterol or cholestyramine in the diet. Each point in this diagram represents the mean result obtained from three flasks incubated under each of these experimental conditions.

acetate, octanoate, or glucose incorporated into sterols was calculated using the specific activities of the substrates present in the incubation buffer solutions, and these rates are given as the nmol of each of these substrates incorporated into DPS per g of tissue per hr (nmol$\cdot$g$^{-1}\cdot$hr^{-1}). However, these latter rates had to be corrected for differential losses of ^{14}C during cholesterol biosynthesis. When using [1-^{14}C]acetate or [1-^{14}C]-octanoate, the incorporation rates were multiplied by 1.50, because only 12 of the 18 carbon atoms in [1-^{14}C]acetyl CoA reach cholesterol. Similarly, when using [U-^{14}C]glucose the incorporation rates were multiplied by 1.50 to correct for loss of 2 of the 6 carbon atoms during the oxidation of pyruvate to acetyl CoA and by 1.33, because only 27 of 36 carbon atoms in [1,2-^{14}C]acetyl CoA reach cholesterol. Furthermore, a second correction term was introduced into these calculations in order to express the incorporation rates as the nmol of C_2 units, i.e., nmol of acetyl CoA units, flowing into DPS from each of the substrates; thus, the incorporation rates obtained when using [1-^{14}C]acetate, [1-^{14}C]octanoate, and [U-^{14}C]glucose were multiplied by the additional factors of 1.0, 4.0, and 2.0, respectively. In summary, in order to calculate what has been designated in this report as the normalized C_2 flux into DPS, the rates of incorporation of [1-^{14}C]acetate, [1-^{14}C]octanoate, and [U-^{14}C]glucose were multiplied by factors of 1.5, 6.0, and 4.0, respectively. By a similar process, normalized C_2 flux rates into CO_2 from the same substrates were obtained using factors of 1.0, 4.0, and 3.0, respectively.

While these normalized C_2 flux rates include corrections for differential losses of ^{14}C during the flow of carbon atoms through the various metabolic pathways, they do not take into account dilution of the specific activity of the intracellular precursor pool of acetyl CoA. Such corrections can only be made where an estimate of the specific activity of the cytosolic acetyl CoA pool giving rise to cholesterol is available. As previously described, in liver slices incubated with relatively high concentrations of octanoate nearly all of the cytosolic acetyl CoA is derived from the rapid intramitochondrial oxidation of this medium chain length fatty acid (17, 18). Other sources of acetyl CoA production are essentially swamped out and, futher, the specific activity of the intracellular acetyl CoA pool can be estimated by measuring the specific activity of the ketone bodies synthesized during the incubation (18, 19). When the specific activity of the ketone bodies is divided by the specific activity that the ketones should theoretically have if the [1-^{14}C]acetyl CoA generated from [1-^{14}C]octanoate was not diluted by acetyl CoA arising from unlabeled substrates, then the relative ketone specific activity is obtained (18). Thus, when normalized C_2 flux rates from [1-^{14}C]octanoate into DPS and CO_2 are divided by the relative ketone body specific activity, absolute rates of DPS synthesis and CO_2 production are obtained; these values are designated as corrected C_2 flux rates into DPS and CO_2 in this report, e.g., in Table 3.

RESULTS

The first group of studies was undertaken to delineate a number of technical problems that became evident when rates of cholesterol synthesis were measured utilizing [^{3}H]water. In initial experiments much higher apparent rates of sterol synthesis were found in several extrahepatic tissues than were previously reported using ^{14}C-labeled substrates (1–3). However, it was also noted that similar, fairly high rates of [^{3}H]water incorporation into DPS were found in these same tissues when incubated at 0°C as when incubated at 37°C and, further, these rates of incorporation appeared to vary with the type of tissue and the amount of slices in each incubation flask. Thus, as shown in the left panel of **Fig. 1,** the apparent rates of incorporation of [^{3}H]water into DPS at 0°C varied in a linear fashion with the amount of tissue

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TABLE 1. Apparent [^{3}H]water incorporation rates into DPS by several tissues at 37°C and 0°C in the presence and absence of metabolic inhibitors

Tissue	Amount Tissue Slices in Flask	Incubation Conditions			
		A. 0°C	B. 37°C	C. 0°C + Inhibitors	D. 37°C + Inhibitors
	mg	*nmol/flask*			
1) Liver	300	14.9 ± 1.5	173.9 ± 61.3	12.1 ± 3.0	17.7 ± 2.2
2) Mid-Gut	300	6.9 ± 0.7	46.9 ± 7.2	6.3 ± 0.5	8.2 ± 0.7
3) Skin	300	10.2 ± 0.8	27.3 ± 1.5	7.9 ± 0.7	11.1 ± 0.8
4) Brain	200	33.1 ± 1.4	31.9 ± 6.5	31.5 ± 3.9	27.4 ± 4.7

In these studies 200 or 300 mg of tissue slices were placed in flasks containing 2.0 ml of Krebs bicarbonate buffer, 16 μmol of Na acetate, and 10 mCi of [^{3}H]water. In addition, 2 μmol of potassium cyanide and 2 μmol of iodoacetamide were added to the flasks from which the data in columns C and D were obtained. The flasks were then incubated in metabolic shakers set at 120 oscillations per min for 1.5 hr at either 0°C or 37°C. The DPS were isolated and the nmol of [^{3}H]water apparently incorporated into sterols by the tissue in each flask were determined. These data represent mean values ±1 SE for tissues obtained from five animals.

cholesterol added to the incubation flask, regardless of whether the cholesterol was derived from liver or brain. This observation was confirmed by the data shown in the middle panel where 300 mg of slices from five different tissues were incubated at 0°C for 1.5 hr with [^{3}H]water. The apparent incorporation rates varied from 7 to 50 nmol/flask and were directly related to the amount of cholesterol present in 300 mg of each of the tissues. That these apparent incorporation rates had no relationship to the potential rate of cholesterol synthesis occurring in the tissues is shown by the data in the right panel. Under circumstances where the rates of cholesterol synthesis (measured at 37°C) varied from 10 to nearly 700 nmol/flask, the apparent incorporation rates of [^{3}H]water at 0°C were constant at approximately 15 nmol/flask. However, when the cholesterol content of the liver slices was essentially doubled by feeding the animals cholesterol, these apparent incorporation rates increased to approximately 30 nmol/flask.

These studies clearly demonstrated that there were nonspecific, relatively high apparent incorporation rates of [^{3}H]water into DPS, and the magnitude of this incorporation varied directly with the absolute amount of tissue cholesterol in the incubation flask but was independent of the inherent rate of sterol synthesis in the tissue. Such apparent incorporation of [^{3}H]water into DPS could have resulted either from the exchange of ^{3}H from water into the sterol molecule through enzymatic or nonenzymatic mechanisms or by binding of [^{3}H]water to the digitonin precipitates during isolation of the DPS.

In order to be certain that this process was not enzymatically mediated, the studies shown in **Table 1** were carried out. As seen in column A, the incorporation rates at 0°C varied from 6.9 to 33.1 nmol/flask and were again related to the amount of cholesterol present in each of the different tissues. At 37°C (column B) there was a significant increase in the incorporation of [^{3}H]water into DPS in the liver and, to lesser degrees, in the mid-gut and skin but not in the brain. It should be noted that if rates of synthesis were judged by the data obtained at 37°C, erroneous results would have been obtained; correct rates of synthesis could be obtained only by subtracting the rates in column A from those in column B. The important point, however, is that inhibiting tissue metabolism with potassium cyanide and iodoacetamide did not significantly decrease the apparent rates of incorporation of [^{3}H]water into DPS at 0°C (column C). It was also possible that the DPS might acquire ^{3}H through nonenzymatic exchange reactions, particularly under the alkaline conditions and high temperatures used to saponify the tissue lipids. However, in other studies it was shown that there was no relationship between the length of saponification time (between 0.5 and 10 hr) and the apparent incorporation rates of [^{3}H]water into DPS, suggesting that such exchange reactions were not quantitatively important.

It was likely, therefore, that these incorporation rates actually represented [^{3}H]water tightly bound to the digitonin precipitates and not ^{3}H incorporated into sterols. That this was the case is demonstrated by the experimental results shown in **Table 2.** As seen in line 1 of Table 2, liver and brain apparently incorporated 15.3 and 55.2 nmol of [^{3}H]water per flask at 0°C, respectively. However, when the digitonides were split with pyridine and the free sterols were extracted into ether (line 3), these values decreased to only 1.6 and 2.1 nmol/flask, respectively. When these ether extracts were taken to dryness and subjected to thin-layer chromatography, even these low incorporation rates disappeared and no radioactive compounds could be detected at any position on the

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TABLE 2. Effect of splitting the sterol digitonides on apparent [^{3}H]water incorporation rates into DPS by liver and brain slices at 0°C and 37°C

Fraction	Liver A. 0°C	Liver B. 37°C	Brain C. 0°C	Brain D. 37°C
	nmol/flask			
1) Digitonin–Sterol Precipitate	15.3 ± 1.2	123.7 ± 4.2	55.2 ± 3.0	49.7 ± 4.5
2) Digitonin Precipitate	2.5 ± 0.5	18.0 ± 2.5	16.5 ± 2.4	24.5 ± 3.2
3) Ether-Soluble Sterol Fraction	1.6 ± 0.2	105.0 ± 3.0	2.1 ± 0.1	1.2 ± 0.1

Three hundred mg of liver and brain slices were placed in flasks containing 2.0 ml of Krebs bicarbonate buffer, 16 μmol of Na acetate, and 10 mCi of [^{3}H]water. The flasks were incubated in metabolic shakers set at 120 oscillations per min for 1.5 hr at either 0°C or 37°C. The DPS were isolated, dissolved in methanol, and an aliquot was assayed for radioactivity; this value was used to calculate the apparent [^{3}H]water incorporation rates into the digitonin–sterol precipitate shown in line 1. The remainder of the methanol solution was taken to dryness under nitrogen and the precipitate was redissolved in pyridine. The digitonin was then precipitated by the addition of diethyl ether and the precipitate was washed twice with additional amounts of ether. Aliquots of both the digitonin precipitate (line 2) and the combined ether washes containing the free sterol (line 3) were assayed for radioactivity. These incorporation rates represent mean values ±1 SE for data obtained in three separate experiments.

thin-layer plates. In contrast, in the flasks containing liver slices incubated at 37°C, 105 of the 123.7 nmol of [^{3}H]water incorporated were recovered in the ether fraction and, when taken to dryness and subjected to thin-layer chromatography, more than 98% of the ^{3}H cochromatographed with authentic cholesterol. Clearly these results indicate that [^{3}H]water was bound to the digitonin precipitates, giving artifactually high incorporation rates.

Two maneuvers were next tested to eliminate this major source of error. The number of acetone washes of the digitonin precipitates was increased from the conventional two to ten; however, this did not reduce the apparent incorporation rates at 0°C. Next the digitonides were dried at 105°C under vacuum for up to 10 hr. This caused only a 30% reduction in the amount of [^{3}H]water bound to the precipitates, so neither of these manipulations was found to be a practical method for dealing with this technical problem.

We concluded from this initial set of investigations that when tissue was incubated with [^{3}H]water there were high backgrounds of radioactivity that could totally obscure the inherent rates of sterol synthesis in a given tissue. This background resulted from [^{3}H]water trapped in or bound to the digitonin precipitates (about 20 nmol of water were bound per 1.0 mg of cholesterol preciptated as the digitonide) in such a way that it could not be easily removed by acetone washing or by heating under vacuum. Accurate incorporation rates, therefore, could be obtained only by running control flasks for each tissue at 0°C and subtracting these values from those obtained at 37°C or, alternatively, by splitting the digitonin precipitates with pyridine and assaying the radioactivity in the extracted and carefully dried free sterols. In all subsequent studies we have used the former method.

With these technical problems clarified it was possible to next undertake the second group of studies designed to experimentally measure the relative rates of incorporation of H and C into the cholesterol molecule under a variety of physiological conditions. Such experiments can be done only under circumstances where absolute flux rates of carbon into cholesterol can be measured, and such measurements, in turn, require correction of the incorporation rates of ^{14}C-labeled substrates into DPS for dilution of the specific activity of the intracellular acetyl CoA pool from which sterols are synthesized. Methodology to make such corrections is currently available only for the liver (18, 19). In order to be certain, however, that the value derived for the H/C incorporation ratio was constant under a variety of physiological and experimental conditions, these measurements were carried out under circumstances where the rates of cholesterol synthesis were varied over a wide range and where the predominant source for the acetyl CoA being incorporated into the cholesterol came from either intracellular or extracellular sources.

As described in detail in **Table 3,** 16 flasks were run for each animal. Liver slices were taken from fed animals (lines 1 and 2), from animals fasted for 24 hr (line 3), and from animals fed cholestyramine to increase the rate of cholesterol synthesis (line 4). In addition to 300 mg of liver slices, each flask contained octanoate at a concentration of either 1 or 2 mM (column A) and either [1-^{14}C]octanoate or [^{3}H]water. Columns B and C give the apparent rates of incorporation of these two labeled compounds into DPS in flasks incubated at either 0°C or 37°C. Column D shows the rates of ketone body synthesis and column E

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TABLE 3. Determination of the ratio of H to C incorporation into the cholesterol molecule during sterol synthesis in the liver

Experiment Group	A. Octanoate Concentration	B. [1-^{14}C]-Octanoate Incorporation into DPS 0°C	B. 37°C	C. [^{3}H]Water Incorporation into DPS 0°C	C. 37°C	D. Total Ketone Synthesis 0°C	D. 37°C	E. Relative Ketone Specific Activity	F. Corrected C_2 Flux into DPS	G. Corrected [^{3}H]Water Flux into DPS	H. H/C Ratio
	mM	*nmol/flask*		*nmol/flask*		*μmol/flask*		*% theoretical*	*nmol/flask*		
1) Fed	2.0	0.11 ± 0.02	19.70 ± 6.42	14.9 ± 2.2	114.1 ± 33.0	0.41 ± 0.13	3.51 ± 0.08	82.0 ± 2.0	143.3 ± 50.5	99.2 ± 26.4	0.69 ± 0.03
2) Fed	1.0	0.02 ± 0.00	20.18 ± 3.83	15.5 ± 1.1	242.6 ± 44.8	0.39 ± 0.05	2.12 ± 0.05	37.0 ± 2.0	325.9 ± 56.7	227.1 ± 44.5	0.69 ± 0.07
3) Fasted	2.0	0.08 ± 0.02	7.30 ± 0.36	18.5 ± 0.6	68.4 ± 4.9	0.44 ± 0.03	7.35 ± 0.23	57.0 ± 1.0	76.0 ± 3.4	49.9 ± 4.9	0.66 ± 0.04
4) Cholestyramine	2.0	0.08 ± 0.01	99.75 ± 6.58	16.5 ± 0.3	583.8 ± 59.7	0.29 ± 0.03	3.23 ± 0.16	76.0 ± 2.0	786.9 ± 37.5	567.3 ± 57.7	0.72 ± 0.05

In these studies 16 flasks were run from each experimental animal; all flasks contained 300-mg aliquots of liver slices in 2.0 ml of Krebs bicarbonate buffer containing either 2 or 4 μmol of Na octanoate (column A). Six of the flasks also contained 10 mCi of [^{3}H]water while the remaining 10 flasks contained [1-^{14}C]octanoate. Three of the flasks containing [^{3}H]water and five of the flasks containing [1-^{14}C]octanoate were incubated at 0°C while the remaining flasks were incubated at 37°C for 1.5 hr in metabolic shakers set at 120 oscillations per min. Column B gives the nmol of [1-^{14}C]octanoate incorporated into DPS in each flask at 0°C and 37°C while column C shows the nmol of [^{3}H]water incorporated into DPS under these experimental conditions. Column D similarly gives the rates of ketone synthesis in the liver slices at these two temperatures. Column E gives the specific activity of the newly synthesized ketones expressed as a percentage of the theoretical specific activity that they would have been expected to have if no intracellular dilution of the acetyl CoA pool had occurred. Column F gives the corrected C_2 flux into DPS calculated from the data in columns B and E while column G gives the corrected [^{3}H]water flux into DPS calculated by subtracting the incorporation rates shown in column C obtained at 0°C from those obtained at 37°C. The ratios shown in column H were obtained by dividing the data in column G by that in column F. The animals in group 1 (five rats) were killed at different points in the light cycle while those in group 2 (three rats) were killed at the mid-dark phase of the light cycle. The animals in group 3 (five rats) were fasted 24 hr before being killed while those in group 4 (four rats) were fed 2% cholestyramine for 7 days before being used in these studies. All data represent mean values ±1 SE.

lists the relative specific activity of the newly synthesized ketone bodies. In each experimental group the corrected C_2 flux from [1-^{14}C]octanoate into DPS (column F) was calculated from the rate of incorporation of this substrate at 37°C minus that at 0°C, the appropriate correction factor (see Methods), and the relative ketone specific activity. The corrected [^{3}H]-water flux into DPS (column G) was calculated from the difference in the apparent incorporation rates at 37°C and 0°C. Since cell water equilibrates with the water in the incubation medium within minutes, no correction was required for differences in the specific activity of water in these two compartments. The ratio of incorporation of H and C into DPS could then be calculated directly from the corrected C_2 flux rates and [^{3}H]water flux rates (column H).

In liver from fed animals incubated with 2 mM octanoate (line 1), the majority of the acetyl CoA pool in the cell was derived from the exogenous substrate, as evidenced by the relative ketone specific activity of 82%. In this circumstance the H/C ratio was 0.69 ± 0.03. When the octanoate concentration was reduced to 1.0 mM (line 2) most of the acetyl CoA units were derived from intracellular sources (relative ketone specific activity of 37%), but the H/C ratio remained at 0.69 ± 0.07. Similary, the H/C ratio equaled 0.66 ± 0.04 in the livers from animals fasted for 24 hr where there is known to be a marked increase in the oxidation of intracellular substrates as evidenced by the decrease in the relative ketone specific activity and the 2-fold increase in the rate of ketone synthesis (line 3 vs. line 1). Finally, when the rate of sterol synthesis was greatly increased by cholestyramine feeding, the H/C ratio equaled 0.72 ± 0.05. The independence of the value of the H/C ratio and the rate of cholesterol synthesis is further emphasized in **Fig. 2** where the corrected C_2 flux rate into DPS and the H/C ratio found in each individual animal is plotted.

From these various studies we concluded that the ratio of H to C incorporation in DPS in the liver was constant and was independent of the rate of the cholesterol synthesis, the rate of acetyl CoA production within the cell, and the source of the substrate from which the acetyl CoA was being produced. Therefore, the mean value for the H/C ratio obtained from the 17 animals in this study (0.69 ± 0.03) was used for all subsequent calculations. It should be emphasized that this ratio gives the μg-atoms of ^{3}H that are incorporated into DPS for each μg-atom of C that enters the cholesterol biosynthetic pathway as acetyl

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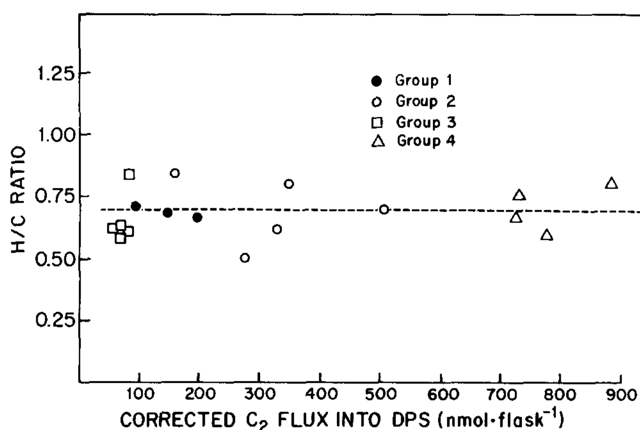

Fig. 2. H/C ratios determined in livers of animals with different rates of sterol synthesis. These experiments are described in detail in the legend to Table 3. In this diagram the H/C ratio obtained in each animal is plotted against the corrected C_2 flux into DPS found in the same liver. The different groups of animals refer to the experimental groups listed in Table 3. Each point represents the result obtained in a single animal.

CoA. This value can also be expressed as the ratio of the μg-atoms of ^{3}H incorporated into the sterol molecule per μg-atom of C present in the final cholesterol molecule. Since 9 of the 36 carbon atoms that enter the biosynthetic pathway are lost, this latter value equals 0.89 ± 0.04.

With this value established, the third set of studies was undertaken where rates of incorporation of [^{3}H]water and three ^{14}C-labeled substrates were measured in parallel in slices obtained from ten different tissues. The manner in which these studies were carried out is illustrated in detail in the case of the liver by the data given in **Table 4.** Aliquots of slices prepared from the liver of each animal were incubated with [^{3}H]water in the presence of unlabeled acetate (8 mM), octanoate (2 mM), or glucose (4 mM); these concentrations were chosen to yield the same potential concentration of acetyl CoA units, i.e., 8 mM C_2 units, upon complete metabolism of the respective substrates. Aliquots of slices also were incubated in parallel in media containing [1-^{14}C]acetate (8 mM), [1-^{14}C]octanoate (2 mM), or [U-^{14}]glucose (4 mM). Column A shows the rate of [^{3}H]water incorporation into DPS in the presence of unlabeled acetate, octanoate, or glucose. These rates multiplied by the reciprocal of the H/C ratio give the theoretical rates of C_2 flux into DPS shown in column B. These latter values presumably represent the absolute rates of acetyl CoA incorporation into DPS under these three experimental conditions. Column C and D, respectively, show the experimental values obtained for the incorporation of each of the ^{14}C-labeled substrates into CO_2 and DPS. When these values are corrected for the differential loss of radioactivity during cholesterol biosynthesis and are normalized to the nmol or μmol of actyl CoA incorporated into DPS and CO_2, the flux rates shown in columns E and F are obtained. The normalized C_2 flux into DPS from each ^{14}C-labeled substrate can then be compared to the theoretical C_2 flux into DPS obtained with [^{3}H]water and expressed as a percentage; these

TABLE 4. Theoretical C_2 flux rates into DPS and normalized C_2 flux rates from [1-^{14}C]acetate, [1-^{14}C]octanoate, and [U-^{14}C]glucose into CO_2 and DPS by the liver

Substrate and Concentration	A. [^{3}H]Water Incorporation into DPS	B. Theoretical C_2 Flux into DPS	^{14}C-Labeled Substrate Incorporated into		Normalized C_2 Flux into		G. $\frac{F}{B} \times 100$
			C. CO_2	D. DPS	E. CO_2	F. DPS	
	nmol·g^{-1}·hr^{-1}	*nmol·g^{-1}·hr^{-1}*	*µmol·g^{-1}·hr^{-1}*	*nmol·g^{-1}·hr^{-1}*	*µmol·g^{-1}·hr^{-1}*	*nmol·g^{-1}·hr^{-1}*	*%*
1) Acetate (8 mM)	448.0 ± 77.0	650.0 ± 112.0	8.49 ± 0.50	193.0 ± 43.0	8.49 ± 0.50	290.0 ± 64.0	44.6 ± 2.0
2) Octanoate (2 mM)	328.0 ± 77.0	476.0 ± 112.0	2.08 ± 0.11	60.5 ± 13.5	8.32 ± 0.44	363.0 ± 81.0	76.3 ± 8.0
3) Glucose (4 mM)	521.0 ± 95.0	755.0 ± 137.0	0.28 ± 0.03	4.3 ± 0.8	0.84 ± 0.09	17.2 ± 3.2	2.3 ± 0.2

In this study 15 flasks were run from each animal: all flasks contained 300-mg aliquots of liver slices in 2.0 ml of Krebs bicarbonate buffer. Four flasks contained either 16 μmol of Na acetate or 8 μmol of D-glucose while seven flasks contained 4 μmol of Na octanoate. Two of the flasks from the groups containing Na acetate, Na octanoate, and D-glucose also contained [1-^{14}C]acetate, [1-^{14}C]octanoate, and [U-^{14}C]glucose, respectively, while an additional pair of flasks from each of these groups contained [^{3}H]water. These 12 flasks were incubated at 37°C for 1.5 hr in metabolic shakers set at 120 oscillations per min. [^{3}H]Water also was added to the remaining three flasks containing Na octanoate and these were incubated at 0°C under the same conditions. After subtracting the apparent incorporation rates of [^{3}H]water into DPS in the three flasks incubated at 0°C, the nmol of [^{3}H]water incorporated into DPS per g of liver slices per hr in the presence of the three substrates was calculated and is shown in column A. These values were then multiplied by the reciprocal of the H/C ratio (1.45) to give the theoretical values for the C_2 flux into DPS shown in column B. Columns C and D give the experimentally determined rates of incorporation of each of the ^{14}C-labeled substrates into CO_2 and DPS while columns E and F give the normalized C_2 flux rates into CO_2 and DPS derived from these experimental values as described in Methods. In column G the normalized C_2 flux rate from each substrate into DPS (column F) is expressed as a percentage of the theoretical C_2 flux (column B). These data represent mean values ±1 SE for determinations in five animals.

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TABLE 5. Normalized C_2 flux rates from [1-^{14}C]acetate, [1-^{14}C]octanoate, and [U-^{14}C]glucose into CO_2 and DPS by different tissues

	Acetate (8 mM)				Octanoate (2 mM)				Glucose (4 mM)			
Tissue	A. Theoretical C_2 Flux into DPS	Normalized C_2 Flux into B. CO_2	Normalized C_2 Flux into C. DPS	D. $\frac{C}{A} \times 100$	E. Theoretical C_2 Flux into DPS	Normalized C_2 Flux into F. CO_2	Normalized C_2 Flux into G. DPS	H. $\frac{G}{E} \times 100$	I. Theoretical C_2 Flux into DPS	Normalized C_2 Flux into J. CO_2	Normalized C_2 Flux into K. DPS	L. $\frac{K}{I} \times 100$
	nmol · g⁻¹ · hr⁻¹	*µmol · g⁻¹ · hr⁻¹*	*nmol · g⁻¹ · hr⁻¹*	%	*nmol · g⁻¹ · hr⁻¹*	*µmol · g⁻¹ · hr⁻¹*	*nmol · g⁻¹ · hr⁻¹*	%	*nmol · g⁻¹ · hr⁻¹*	*µmol · g⁻¹ · hr⁻¹*	*nmol · g⁻¹ · hr⁻¹*	%
1) Liver	650.0 ± 112.0	8.49 ± 0.50	290.0 ± 64.0	44.6 ± 2.0	476.0 ± 112.0	8.32 ± 0.44	363.0 ± 81.0	76.3 ± 8.1	755.0 ± 137.0	0.84 ± 0.09	17.2 ± 3.2	2.3 ± 0.2
2) Small bowel	90.5 ± 24.8	4.45 ± 0.60	43.8 ± 7.8	48.4 ± 3.4	72.7 ± 16.9	9.03 ± 1.08	66.3 ± 14.8	91.2 ± 13.0	118.2 ± 25.6	8.42 ± 1.13	31.4 ± 6.8	26.6 ± 1.8
3) Skin	37.1 ± 5.6	0.45 ± 0.04	2.6 ± 0.5	7.0 ± 0.3	34.2 ± 6.2	1.19 ± 0.18	11.8 ± 3.0	34.5 ± 3.5	54.2 ± 11.9	1.69 ± 0.16	15.9 ± 2.9	29.3 ± 2.4
4) Kidney	17.5 ± 2.1	27.06 ± 0.48	10.8 ± 0.4	61.7 ± 6.0	19.8 ± 2.2	23.69 ± 0.58	17.9 ± 2.1	90.4 ± 10.4	13.3 ± 1.6	14.97 ± 0.56	11.7 ± 2.9	88.0 ± 2.1
5) Spleen	9.5 ± 2.0	3.95 ± 0.17	4.1 ± 0.8	43.2 ± 11.2	7.4 ± 1.4	6.97 ± 0.29	4.9 ± 0.6	66.2 ± 7.3	13.8 ± 1.5	5.05 ± 0.13	5.6 ± 0.4	40.6 ± 2.4
6) Adrenal gland	1682.5 ± 191.9	1.06 ± 0.03	67.4 ± 9.8	4.0 ± 1.4	1569.9 ± 253.6	4.78 ± 0.32	206.4 ± 75.5	13.1 ± 2.1	2445.8 ± 498.5	0.17 ± 0.01	39.6 ± 10.1	1.6 ± 1.3
7) Ovary	746.7 ± 99.7	2.84 ± 0.23	131.0 ± 15.9	17.6 ± 3.1	1110.8 ± 129.1	2.59 ± 0.15	167.2 ± 30.8	15.1 ± 2.4	1131.1 ± 253.9	0.34 ± 0.02	123.6 ± 24.2	10.9 ± 1.4
8) Testis	23.5 ± 2.1	1.26 ± 0.17	8.0 ± 1.3	34.0 ± 6.1	18.4 ± 2.0	1.00 ± 0.08	5.1 ± 1.7	27.7 ± 6.3	38.5 ± 4.4	5.84 ± 0.47	5.2 ± 1.4	13.5 ± 2.1
9) Lung	11.2 ± 1.6	1.57 ± 0.18	2.8 ± 0.5	25.0 ± 3.6	9.6 ± 1.6	4.40 ± 0.45	3.0 ± 0.7	31.3 ± 3.1	16.4 ± 4.2	2.98 ± 0.28	3.7 ± 0.3	22.6 ± 3.8
10) Muscle	1.5 ± 0.3	0.08 ± 0.00	0.1 ± 0.0	6.7 ± 2.6	1.7 ± 0.9	0.23 ± 0.02	0.1 ± 0.0	5.9 ± 2.8	0.4 ± 0.2	0.07 ± 0.00	0.2 ± 0.0	50.0 ± 5.2

The details of how each of these experiments was undertaken and how the various values were calculated are given in the legend to Table 4. In this table only the final calculated values are given for the theoretical C_2 flux into DPS based on the rates of incorporation of [^{3}H]water, assuming the H/C ratio determined in liver applies to these tissues, and the normalized C_2 flux into CO_2 and DPS based on incorporation of the three ^{14}C-labeled substrates. All tissues were obtained from fed, control rats except the adrenal gland and ovary which were obtained from animals treated with APP. Each value in this table represents the mean ± 1 SE for 4–6 experiments with each tissue.

values are given in column G. As is apparent, the results obtained are very different with the various substrates. Octanoate gives 76.3% of the theoretical rates of sterol synthesis. This value is expected from previously reported data indicating that under these experimental conditions octanoate rapidly penetrates the cell membranes and is oxidized to acetyl CoA units so that there is only about a 24% dilution of the specific activity of the acetyl CoA pool by the generation of acetyl CoA from unlabeled, intracellular substrates (18). In contrast, acetate and glucose give only 44.6% and 2.3% of the theoretical rates, respectively. Presumably these marked differences are due to differences in the rates of cell entry or metabolism to acetyl CoA or to differences in dilution of the specific activity of the substrate within the cell, e.g., as may occur when the [U-^{14}C]glucose enters the cell and is mixed with unlabeled glucose arising from the breakdown of glycogen.

Identical studies were carried out in nine other tissues and the results are summarized in **Table 5.** It should be noted that all of these tissues, except in the adrenal gland and ovary, were obtained from fed, control animals. The two endocrine tissues, however, were taken from APP-treated animals in order to increase the rates of sterol synthesis in these two organs where the amount of tissue available for incubation was very limited (4, 14). Only the theoretical C_2 flux (from [^{3}H]water incorporation rates) and the normalized C_2 flux from each of the ^{14}C-labeled substrates into CO_2 and DPS are presented in this table.

It is apparent that there are striking differences of four types in the rates of synthesis in these tissues measured with the various substrates. First, in the same tissue the normalized C_2 flux rates from acetate, octanoate, and glucose (columns C, G, and K) were similar for such tissues as lung, testes, ovary, and

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spleen, but there was a 5- to 20-fold difference in the rates measured with these substrates in such tissues as liver, skin, and adrenal gland.

Second, there was also marked variability in the extent to which the C_2 flux from each of these substrates reflected the theoretical C_2 flux in each tissue. For example, in ovary and lung, normalized C_2 flux rates with acetate, octanoate, and glucose gave similar percentages of the theoretical rates found in each respective tissue. In skin, kidney, and testis, however, there was agreement between only two of the [^{14}C]substrates while in such tissues as liver and adrenal gland there was no similarity at all in the degree to which the normalized C_2 flux rates determined with acetate, octanoate, and glucose approximated the theoretical C_2 flux rates.

Third, for any ^{14}C-labeled substrate there were major differences in the degree to which its incorporation rate reflected the theoretical rates in different tissues. Octanoate gave rates that exceeded 90% of the theoretical values in the small bowel and kidney but that equaled over 20% of the rates found in adrenal gland, ovary, and muscle; even greater variability was found with acetate and glucose.

Fourth, even the theoretical rates varied in the same tissue depending upon which substrate was present in the incubation medium. Thus, as is also apparent in Table 5, in several tissues such as liver and small bowel the highest theoretical C_2 flux rates were found when glucose was added to the medium while lower absolute rates were detected in the presence of acetate or octanoate. In other tissues, however, acetate or octanoate supported the highest theoretical rates of DPS synthesis.

Thus, from these studies it is obvious that the apparent rates of synthesis measured in any tissue under in vitro conditions are influenced nearly as much by the substrate used or the particular additions to the incubation medium as by the inherent rates of sterol synthesis. The major generalization that can be made from these data, however, is that previously reported rates of synthesis in extrahepatic tissues represent gross underestimates of the true rates of sterol synthesis in these various organ systems.

DISCUSSION

In dealing with problems related to physiological regulation of plasma cholesterol levels in health and in various diseases, it is obviously critically important to be able to quantitate the rates of sterol synthesis in the liver and in a variety of extrahepatic tissues under in vivo conditions. While several papers have reported attempts to make such measurements in the liver and intestine (20, 21), there has as yet not been a comprehensive evaluation of the numerous potential technical and theoretical problems inherent in such measurements. To circumvent some of these problems, most measurements of rates of cholesterol synthesis have been made under in vitro conditions using either whole cell preparations like tissue slices or isolated cells, or in tissue homogenates or isolated microsomes. Each of these preparations has certain advantages and disadvantages and each method, under the appropriate conditions, probably gives valid *relative* rates of cholesterol synthesis in a given tissue. However, these various methods almost certainly do not yield valid *absolute* rates of synthesis under in vitro conditions, and may not allow direct comparisons, therefore, between the rates of cholesterol synthesis in different tissues.

Since there is now considerable interest in the regulation of synthesis in extrahepatic tissues as well as in the liver (3, 4, 22, 23), it becomes particularly important to be able to measure absolute rates of synthesis in a variety of tissues under circumstances where differences in rates of cell penetration and metabolism of radiolabeled substrates and differences in the degree of dilution of the specific activity of the precursor pools within the tissues do not introduce significant errors into the measurements. In a number of respects, [^{3}H]water should be better than most ^{14}C-labeled substrates for quantitating rates of cholesterol synthesis in various tissues. For example, extracellular water rapidly penetrates most cell membranes and so equilibrates with intracellular water within minutes; [^{3}H]water does not require extensive metabolism prior to incorporation into the sterol molecule; and there is little likelihood that the specific activity of the intracellular water pool will be diluted by the metabolic generation of unlabeled water. On the other hand, there are at least three major disadvantages to the use of [^{3}H]water. First, because of the large mass of water in the incubation medium, 10^4 to 10^5 times more radioactivity is required in each incubation flask than when ^{14}C-labeled substrates are used. This necessitates special laboratory precautions and all studies and analytic procedures must be carried out under appropriate fume hoods. Second, as shown by the initial group of studies reported here, it is necessary to correct all incorporation rates for carry-over of significant amounts of [^{3}H]water with the digitonin precipitates. Since such carry-over is proportional to the cholesterol content of a given tissue, failure to make such corrections will have relatively little effect on rates of synthesis in active tissues with a relatively low cholesterol content, such as liver, but will result in significant errors in

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other tissues with lower rates of synthesis or higher cholesterol levels. Third, in order to obtain absolute rates of cholesterol synthesis after measuring rates of [^{3}H]water incorporation into sterols, one must have an accurate value defining the μg-atoms of C incorporated into the cholesterol molecule per μg-atom of H.

Recently Lakshmanan and Veech (24) reviewed the pathways by which ^{3}H from [^{3}H]water can be incorporated into the sterol nucleus and, from these considerations, the theoretical values of the H/C incorporation ratio can be calculated. ^{3}H atoms may be incorporated into the cholestrol molecule either directly from [^{3}H]water or during certain reductive steps involving NADPH. The degree to which NADPH becomes labeled with ^{3}H in the presence of [^{3}H]water, in turn, depends to some extent upon the source of the reduced diphosphopyridine nucleotide; NADPH derived from the pentose cycle does not become labeled with ^{3}H while that derived from other oxidative enzymatic reactions does equilibrate with [^{3}H]water in the medium. During the biosynthesis of cholesterol, 18 μmol of acetyl CoA give rise to 1 μmol of cholesterol containing 27 μg-atoms of C and 46 μg-atoms of H. Of these 46 μg-atoms of H, 15 are derived from NADPH and 7 come directly from water in the medium. Thus, the number of μg-atoms of ^{3}H incorporated into cholesterol will depend upon the degree of equilibration of the H of NADPH with [^{3}H]water. From these theoretical considerations the two extreme values that would be possible for the H/C incorporation ratio can be derived. Under circumstances where none of the H of NADPH becomes labeled with ^{3}H, only 7 μg-atoms of ^{3}H will be incorporated into cholesterol, giving an H/C ratio of only 0.26. In contrast, if the H of NADPH fully equilibrates with [^{3}H]water, then the sterol molecule will contain 22 μg-atoms of ^{3}H and yield a theoretical value for the H/C ratio of 0.81. On the basis of available information it is difficult to predict which of these values would necessarily apply to the liver under either in vivo or in vitro conditions, so it is clearly necessary to experimentally determine the H/C ratio under the conditions that one is using. The difficulty, however, with such experimental measurements is that corrections must be made for dilution of the specific activity of the [^{14}C]acetyl CoA pool that is generated from a particular ^{14}C-labeled substrate so that absolute rates of carbon incorporation into cholesterol can be determined. This problem has been dealt with in different ways by several different investigators.

In the present study, the generation of acetyl CoA was driven by incubation of liver slices with 2 mM [1-^{14}C]octanoate and the small amount of residual dilution of the specific activity of the [1-^{14}C]acetyl CoA was corrected for by using the specific activity of the newly synthesized ketone bodies (18). On the basis of these measurements in the liver slices obtained from animals subjected to a variety of physiological manipulations (Table 3) an average of 0.69 μg-atoms of H were incorporated into the sterol molecule for each μg-atom of C that entered the biosynthetic pathway as acetyl CoA. From this value it can be calculated that 24 μg-atoms of ^{3}H were incorporated into the cholesterol molecule, giving a final H/C ratio of 0.89.

Similar values for the H/C ratio in the rat have been obtained in other laboratories using more indirect techniques. Bruengraber et al. (25), for example, measured the incorporation of [^{14}C]glucose and [^{3}H]water into both fatty acids and cholesterol in the isolated perfused liver of the rat. These authors assumed that deviation of the H/C incorporation ratio in long chain fatty acids from the published value of 0.87 (26) was due to dilution of the specific activity of the acetyl CoA pool generated from the [^{14}C]glucose. After correcting for this dilution, it can be calculated from their results that approximately 21 μg-atoms of ^{3}H were present in each μmol of cholesterol, giving a final H/C ratio of 0.76.

In two other studies rats were fed water enriched with either [^{3}H]water (27) or deuterium (28) for prolonged periods of time. The specific activity of the H derived for hepatic cholesterol proved to be only 55% and 50%, respectively, of the specific activity of the H of body water in these two studies. From these data it can be calculated that either 25 μg-atoms of ^{3}H (the [^{3}H]water feeding experiment) or 23 μg-atoms of D (the deuterium-labeled water feeding experiment) entered the sterol molecule from body water, giving final H/C incorporation ratios of 0.93 and 0.85, respectively.

Thus, from these four different experiments from 21 to 25 μg-atoms of H were incorporated into cholesterol from water, giving H/C ratios that varied from 0.76 to 0.93 and that averaged 0.86. Clearly these values are all consistent with the theoretical values calculated above for the situation where the reductive H of NADPH is fully equilibrated with the ^{3}H of [^{3}H]water in the incubation medium, i.e., 22 μg-atoms of ^{3}H should be incorporated into the cholesterol molecule, giving a H/C ratio of 0.81. It should also be emphasized that a similar conclusion was reached by Foster and Bloom (29); using the labeling pattern of the hydrogen atoms in long chain fatty acids synthesized in slices of rat liver, these authors concluded that the H of NADPH must be fully equilibrated with the [^{3}H]water in the medium.

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There are two other reports in the literature, however, of the finding of significantly lower values for the H/C ratio. In one study Barth, et al. (30) attempted to measure the absolute incorporation rate of carbon atoms into cholesterol from [^{14}C]acetate in the isolated perfused liver; the specific activity of the newly synthesized ketones was used to correct for dilution of the specific activity of the acetyl CoA pool in the liver cell. However, in contrast to the situation where [^{14}C]-octanoate is used as the carbon precursor, [^{14}C]acetate enters directly into the cytosolic compartment where cholesterol biosynthesis takes place. [^{14}C]Acetate also reaches the intramitochondrial pool of acetyl CoA from which ketone bodies are synthesized, but the specific activity of this pool presumably undergoes further dilution by the oxidation of fatty acids and other unlabeled substrates. Hence, it is very likely that, in this situation, use of the specific activity of ketones to correct for dilution of the specific activity of the cytosolic acetyl CoA pool will lead to overestimation of the flow of C into cholesterol from acetate and, consequently, to an erroneously low H/C ratio (18). It is not surprising, therefore, that these authors reported that only 9.5 μg-atoms of ^{3}H were apparently incorporated into each μmol of cholesterol, giving a H/C ratio of 0.35. In a more recent publication Lakshmanan and Veech (24) reported an intermediate value for the H/C ratio in the liver of 0.53 (an apparent incorporation of only 14.5 μg-atoms of ^{3}H into each μmol of cholesterol). These values, however, were based upon the measurement of [^{14}C]mevalonate incorporation into cholesterol and on the assumption that the reductive H of NADPH achieved only about half the specific activity of ^{3}H from [^{3}H]water in the medium. Other data, as discussed above, suggest that this is not the case. It seems likely to us, therefore, that both of these latter values for the H/C ratio are too low.

A fundamental assumption inherent in these studies is that the value of the H/C ratio determined in vitro in liver is also applicable to the biosynthesis of cholesterol under similar conditions in other tissues. This value would be expected to vary, for example, in the circumstances where the digitonin-precipitable sterols synthesized by a particular tissue contained significant amounts of 3β hydroxysterols other than cholesterol. Three lines of evidence, however, suggest that this would be a minor source of error in these calculations. First, in nearly all extrahepatic tissues, as in the liver, cholesterol is the major sterol that is synthesized under such in vitro conditions (1); the skin and endocrine glands are the major exceptions (1). Second, the value of the H/C ratio should vary little from 0.89, even in those tissues that synthesize significant amounts of other sterols such as lanosterol, cholestenol, and the methostenols, since variation in these terminal enzymatic reactions will alter only slightly the quantitative relationships between the numbers of C and H atoms incorporated into the basic sterol structure. Third, as discussed above, long-term feeding experiments have yielded H/C incorporation ratios of 0.93 and 0.85 (27, 28). Since rapid equilibration of cholesterol occurs between most tissue compartments under such long-term, in vivo conditions, these values can be considered as "whole body" H/C ratios. The fact that they agree so closely with the value of 0.89 found for the liver in the present study further suggests that the H/C ratio in the sites of sterol synthesis in extrahepatic tissues must be very similar.

Apart from these somewhat theoretical considerations, these studies have important practical implications with respect to the measurement of rates of synthesis in many extrahepatic tissues. It is clear from the results shown in Table 5 that the importance of extrahepatic synthesis has been grossly underestimated in previous in vitro studies. When acetate is used as a substrate, for example, the rates of synthesis are, at best, only about half of the true rates, e.g., in liver, small bowel, kidney, and spleen, and, at worst, they equal less than 10% of the actual rates of sterol synthesis, e.g., in skin, adrenal gland, and muscle. Similar marked discrepancies exist when either octanoate or glucose is used as the radiolabeled precursor, although the magnitude of the error varies with both the substrate used and the tissue tested.

There are at least three reasons why these highly variable errors are seen with different ^{14}C-labeled substrates in the different tissues. In some cases the rate of cell membrane penetration of the precursor may be rate limiting. In others, a particular cell may not have the enzymatic pathways to rapidly metabolize a precursor molecule to acetyl CoA. Finally, and perhaps most importantly, the specific activity of either the substrate itself or the acetyl CoA generated from that substrate may be diluted within the cell. All three of these effects can probably be discerned in the different tissues in the present study. For example, the fact that [1-^{14}C]acetate yields rates of cholesterol synthesis in liver that are only about 45% of the theoretical rates clearly is due to dilution of the cytosolic acetyl CoA pool that is utilized for sterol synthesis by acetyl CoA generated from unlabeled substrates within the mitochondria (18). In contrast, the fact that [U-^{14}C]glucose gives only 2% of the theoretical rate of cholesterol synthesis and that CO_2 production from this substrate is only about 10% of that seen with acetate or octanoate is consistent with the view that the specific activity of the precursor itself undergoes marked dilu-

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tion within the cell, probably through the breakdown of glycogen and generation of large amounts of unlabeled glucose. As is also apparent in Table 5, the kidney is capable of oxidizing all three substrates to CO_2 at very high rates, indicating not only that acetate, octanoate, and glucose can rapidly enter the renal cell under these in vitro conditions but that this tissue is also capable of rapidly metabolizing these three substrates to acetyl CoA. It is not surprising, therefore, that these three ^{14}C-labeled substrates all yield rates of sterol synthesis that are in the range of 62–90% of the theoretical rates. In contrast, in a tissue like skin, the three ^{14}C-labeled substrates give low rates of CO_2 production as well as low rates of cholesterol synthesis. This finding suggests that either the rate of cell penetration (relative to [^{3}H]water) or the rate of metabolism to acetyl CoA is relatively limiting in such tissues.

On the basis of these studies it is apparent that there are at least four general methods available whereby these problems can be overcome so that more accurate absolute rates of sterol synthesis can be measured in whole cell preparations from different tissues under in vitro conditions. First, rates of [^{3}H]water incorporation into sterols can be measured and these rates can then be converted to rates of acetyl CoA incorporation utilizing the H/C incorporation ratio. This method is generally applicable to all tissues but necessarily involves use of large amounts of a volatile radioactive precursor.

Second, an appropriate ^{14}C-labeled substrate can be selected for use in a particular tissue provided it can be shown that this substrate gives rates of sterol synthesis that are close to 100% of the rates obtained with [^{3}H]-water. In this study, for example, octanoate yielded rates of synthesis in small bowel and kidney that were over 90% of the theoretical rates and that were in the range of 65–80% of the theoretical rates in other tissues like liver and spleen. Similarly, in kidney, either [^{14}C]glucose or [^{14}C]acetate give rates that are reasonably close to rates obtained with [^{3}H]water. Unfortunately, it is also evident that in the majority of extrahepatic tissues none of the ^{14}C-labeled substrates tested yielded rates of synthesis that were close to the rates found with [^{3}H]water.

Third, under certain circumstances it is possible to correct rates of incorporation of a ^{14}C-labeled substrate into cholesterol for intracellular dilution of the specific activity of the acetyl CoA pool from which the sterols were synthesized (12, 18, 19). This method has been used extensively in measuring rates of hepatic cholesterol synthesis but requires measurement of the specific activity of another product derived from the same acetyl CoA pool. This method, therefore, is cumbersome to perform and has limited applicability to other tissues.

A final method that would be more generally applicable to all of the tissues would be to use one of the ^{14}C-labeled precursors and then correct mathematically the incorporation rates of this substrate into DPS to absolute rates of incorporation using the appropriate ratio given in columns D, H, and L of Table 5. Such a method would be valid, however, only under circumstances where it had been shown that the ratio remained constant in a particular tissue under all physiological circumstances where the studies were being carried out. It should also be pointed out that alterations of variables in the in vitro incubation procedure such as time of incubation, amount of tissue incubated in each flask, volume of buffer added to each incubation flask, tissue slice thickness, shaking rate of the metabolic incubator, and substrate concentration will alter the results and thus need to be optimized for each tissue and substrate.

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REFERENCES

1. Dietschy, J. M., and M. D. Siperstein. 1967. Effect of cholesterol feeding and fasting on sterol synthesis in seventeen tissues of the rat. *J. Lipid Res.* **8:** 97–104.
2. Dietschy, J. M., and J. D. Wilson. 1968. Cholesterol synthesis in the squirrel monkey: Relative rates of synthesis in various tissues and mechanisms of control. *J. Clin. Invest.* **47:** 166–174.
3. Andersen, J. M., and J. M. Dietschy. 1977. Regulation of sterol synthesis in 16 tissues of rat. *J. Biol. Chem.* **252:** 3646–3651.
4. Andersen, J. M., and J. M. Dietschy. 1977. Regulation of sterol synthesis in 15 tissues of rat. *J. Biol. Chem.* **252:** 3652–3659.
5. Srere, P. A., I. L. Chaikoff, and W. G. Dauben. 1948. The in vitro synthesis of cholesterol from acetate by surviving adrenal cortical tissue. *J. Biol. Chem.* **176:** 829–833.
6. Gould, R. G. 1951. Lipid metabolism and atherosclerosis. *Am. J. Med.* **11:** 209–227.
7. Dietschy, J. M., and J. D. Wilson. 1970. Regulation of cholesterol metabolism. *N. Engl. J. Med.* **282:** 1128–1138.
8. Muroya, H., H. S. Sodhi, and R. G. Gould. 1977. Sterol synthesis in intestinal villi and crypt cells of rats and guinea pigs. *J. Lipid Res.* **18:** 301–308.
9. Shefer, S., S. Hauser, V. Lapar, and E. H. Mosbach. 1972. HMG CoA reductase of intestinal mucosa and liver of the rat. *J. Lipid Res.* **13:** 402–412.

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10. Nordstrom, J. L., V. W. Rodwell, and J. J. Mitschelen. 1977. Interconversion of active and inactive forms of rat liver hydroxymethylglutaryl-CoA reductase. *J. Biol. Chem.* **252:** 8924–8934.
11. Brown, M. S., J. L. Goldstein, and J. M. Dietschy. 1979. Active and inactive forms of 3-hydroxy-3-methylglutaryl CoA reductase in the liver of the rat. *J. Biol. Chem.* **254:** 5144–5149.
12. Weis, H. J., and J. M. Dietschy. 1975. The interaction of various control mechanisms in determining the rate of hepatic cholesterogenesis in the rat. *Biochim. Biophys. Acta* **398:** 315–324.
13. Weis, H. J., and J. M. Dietschy. 1969. Failure of bile acids to control hepatic cholesterogenesis: Evidence for endogenous cholesterol feedback. *J. Clin. Invest.* **48:** 2398–2408.
14. Andersen, J. M., and J. M. Dietschy. 1978. Relative importance of high and low density lipoproteins in the regulation of cholesterol synthesis in the adrenal gland, ovary and testis of the rat. *J. Biol. Chem.* **253:** 9024–9032.
15. Sperry, W. M. 1963. Quantitative isolation of sterols. *J. Lipid Res.* **4:** 221–225.
16. Brown, J. L., and J. M. Johnston. 1962. Radioassay of lipid components separated by thin-layer chromatography. *J. Lipid Res.* **3:** 480–481.
17. McGarry, J. D., and D. W. Foster. 1971. The regulation of ketogenesis from octanoic acid. *J. Biol. Chem.* **246:** 1149–1159.
18. Dietschy, J. M., and J. D. McGarry. 1974. Limitations of acetate as a substrate for measuring cholesterol synthesis in liver. *J. Biol. Chem.* **249:** 52–58.
19. Dietschy, J. M., and M. S. Brown. 1974. Effect of alterations of the specific activity of the intracellular acetyl CoA pool on apparent rates of hepatic cholesterogenesis. *J. Lipid Res.* **15:** 508–516.
20. Edwards, P. A., H. Muroya, and R. G. Gould. 1972. In vivo demonstration of the circadian rhythm of cholesterol biosynthesis in the liver and intestine of the rat. *J. Lipid Res.* **13:** 396–401.
21. Fears, R., and B. Morgan. 1976. Studies on the response of cholesterol biogenesis to feeding in rats. Evidence against the existence of diurnal rhythms. *Biochem. J.* **158:** 53–60.
22. Balasubramaniam, S., J. L. Goldstein, J. R. Faust, and M. S. Brown. 1976. Evidence for regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity and cholesterol synthesis in nonhepatic tissues of rat. *Proc. Natl. Acad. Sci. USA* **73:** 2564–2568.
23. Balasubramaniam, S., J. L. Goldstein, and M. S. Brown. 1977. Regulation of cholesterol synthesis in rat adrenal gland through coordinate control of 3-hydroxy-3-methylglutaryl coenzyme A synthase and reductase activities. *Proc. Natl. Acad. Sci. USA* **74:** 1421–1425.
24. Lakshmanan, M. R., and R. L. Veech. 1977. Measurement of rate of rat liver sterol synthesis in vivo using tritiated water. *J. Biol. Chem.* **252:** 4667–4673.
25. Brunengraber, H., J. R. Sabine, M. Boutry, and J. M. Lowenstein. 1972. 3-β-Hydroxysterol synthesis by the liver. *Arch. Biochem. Biophys.* **150:** 392–396.
26. Jungas, R. L. 1968. Fatty acid synthesis in adipose tissue incubated in tritiated water. *Biochemistry* **7:** 3708–3717.
27. Loud, A. V., and N. L. R. Bucher. 1958. The turnover of squalene in relation to the biosynthesis of cholesterol. *J. Biol. Chem.* **233:** 37–41.
28. Alfin-Slater, R. B., H. J. Deuel, Jr., M. C. Schotz, and F. K. Shomoda. 1950. Univ. S. Calif., Consolidated Eng. Corp., Group Report No. N3.
29. Foster, D. W., and B. Bloom. 1963. The synthesis of fatty acids by rat liver slices in tritiated water. *J. Biol. Chem.* **238:** 888–892.
30. Barth, C., M. Liersch, J. Hackenschmidt, H. Ullmann, and K. Decker. 1972. Cholesterol biosynthesis in the isolated perfused rat liver. *Hoppe-Seyler's Z. Physiol. Chem.* **353:** 1085–1093.

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