Effect of alterations of the specific activity of the intracellular acetyl CoA pool on apparent rates of hepatic cholesterogenesis

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Abstract We have previously shown significant dilution of the specific activity of the intracellular acetyl CoA pool when radiolabeled acetate is used as the precursor in liver slice experiments. In the present study, using liver from animals subjected to various manipulations known to alter the rate of cholesterogenesis, the specific activity of the intramitochondrial acetyl CoA pool was 27–49% of the theoretical specific activity expected if no endogenous dilution occurred. Because the cytosolic acetyl CoA pool that gives rise to cholesterol is not in equilibrium with the intramitochondrial pool, these values cannot be used to correct the flux of labeled carbon from [^{14}C]acetate into cholesterol. However, because [^{14}C]octanoate is rapidly oxidized intramitochondrially to acetyl CoA, which feeds both the intra- and extramitochondrial metabolic pathways, [^{14}C]octanoate can be utilized to determine true flux rates of C_2 units into cholesterol and other products. Using this substrate in liver slices from animals subjected to a variety of experimental manipulations, the specific activity of the intracellular acetyl CoA pool was 54–71% of the expected specific activity. After correction for endogenous dilution, the C_2 flux into cholesterol varied from 335 to 459 nmoles·g^{-1}·hr^{-1} in control animals, was suppressed 10–40-fold in animals subjected to fasting and cholesterol feeding, and increased into the range of 1500 nmoles·g^{-1}·hr^{-1} after derepression with cholestyramine feeding or biliary diversion. Data also are presented that show very good agreement between the corrected C_2 flux rate from octanoate into cholesterol and microsomal HMG CoA reductase activity in the same liver under conditions in which the synthetic rates were varied over a 100-fold range.

Supplementary key words substrate dilution · control of cholesterogenesis

The assessment of rates of cholesterol synthesis in various tissues, particularly in liver and intestine, commonly has been undertaken by measuring the rates of incorporation of radiolabeled acetate into sterols by tissue slices or by direct assay of 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase, EC 1.1.1.34), the rate-limiting enzyme in the cholesterogenic pathway (1–5). Although current methods allow relatively easy and rapid measurement of microsomal HMG CoA reductase activity, the slice preparation still has the significant advantage of allowing simultaneous assessment of the rates of cholesterol synthesis and of other metabolic pathways in the cell. This capability is particularly critical in the study of the specific mechanisms operative in the control of cholesterol synthesis because this process is very sensitive to caloric intake as well as to the size of the bile acid pool, stress, the cholesterol content of the diet, diurnal variation in light, and the presence of biliary obstruction (5–7).[1] Hence, it is useful, if not mandatory, to measure rates of CO_2 production, fatty acid synthesis, ketone synthesis, or other metabolic pathways in order to determine if a given experimental manipulation alters hepatic cholesterol synthesis in a specific or nonspecific manner.

When using radiolabeled substrates such as acetate in the whole cell preparation, it is assumed that the rate of penetration of the substrate into the cell and its activation to acetyl CoA is not rate limiting and that the rate of activation of exogenous, radiolabeled acetate is much greater than the rate of production of unlabeled acetyl CoA from endogenous substrates so that the specific activity of the intracellular acetyl CoA pool essentially equals that of the radiolabeled substrate. If this is not the case, the absolute rate of cholesterol synthesis may be grossly underestimated. Furthermore, if the degree of endogenous acetyl CoA production varies with different experimental manipulations, then the apparent rate of incorporation of radiolabeled acetate into cholesterol may vary in a manner that primarily reflects changes in specific activity of the acetyl

Abbreviations: HMG, hydroxymethylglutaryl.

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CoA pool rather than the activity of HMG CoA reductase.

If significant endogenous dilution of the precursor does occur, true rates of sterol synthesis can be obtained in whole cell preparations by correcting the observed rate of radioactivity incorporated into cholesterol for changes in the specific activity of the intracellular acetyl CoA pool. However, as we have recently shown, the situation is even more complex because there are at least two intracellular pools of acetyl CoA (8). When acetate is used as the radiolabeled precursor, the extramitochondrial acetyl CoA pool that gives rise to cholesterol apparently is not in equilibrium with the intramitochondrial pool because acetate penetrates mitochondria relatively slowly and so does not equilibrate with acetyl CoA being generated from the oxidation of endogenous substrates such as fatty acids. Because of this, one cannot use alterations of the specific activity of some product of mitochondrial acetyl CoA metabolism such as CO_2 or ketones to correct the flux rates of radiolabeled acetate into cholesterol for endogenous dilution. To circumvent this problem, we recently have begun using radiolabeled octanoate as the substrate for assessment of rates of cholesterol synthesis in tissue slices (8). This precursor has the advantage of rapidly penetrating the mitochondria, where it generates large amounts of acetyl CoA so that the relative contribution of unlabeled C_2 units from endogenous sources to the total C_2 pool of the cell is greatly reduced. In addition, since in this situation the mitochondrial acetyl CoA pool feeds both intra- and extramitochondrial synthetic pathways, it is possible to correct the flux of the radiolabeled precursor into cholesterol for variations in specific activity of the acetyl CoA pool caused by changes in the rate of conversion of endogenous substrate to unlabeled acetyl CoA.

Since many previous studies have used acetate as the precursor for assessing rates of cholesterol synthesis in studies designed to elucidate the mechanisms of control of the cholesterogenic pathways, we have undertaken the present studies to investigate three specific problems. First, studies are presented that illustrate the degree of variation in dilution of the intramitochondrial acetyl CoA pool that is brought about by various experimental manipulations, e.g., fasting, biliary diversion, and lymphatic diversion, under circumstances where labeled acetate is used as the precursor. Second, using octanoate as the radiolabeled substrate and adjusting for intramitochondrial dilution, corrected rates of cholesterol synthesis have been determined in liver slices under a similar variety of situations known to alter hepatic cholesterogenesis. Third, a direct comparison is made between the corrected rates of cholesterol synthesis using radiolabeled octanoate as the precursor and activity of HMG CoA reductase in liver obtained from animals subjected to various experimental manipulations.

METHODS

Animals

170–230-g female Sprague-Dawley-derived rats (Simonsen Laboratories, Gilroy, Calif.) were used in these studies. All animals were maintained for at least 1 wk on Formulab chow (Ralston Purina Co., St. Louis, Mo.) and water ad lib. prior to use. In certain experiments, as designated in the Results section, animals were exposed to continuous light or to 12-hr cycles of light and dark. In the latter situation the rats were placed in light-tight chambers with forced-air ventilation; lighting in these chambers was controlled by timers and changed at 0300 and 1500. By this means, animals were available at 0900 that were in either the mid-dark or mid-light period of the cycle, i.e., 6 hr after the onset of the dark or light cycle, respectively. In some experiments, animals were operated on for placement of an indwelling gastric cannula as previously described (4). Certain groups of these animals also were subjected to external biliary diversion, external intestinal lymphatic diversion, or biliary obstruction (4). After surgery these rats were placed in restraining cages during the remaining 48 hr of the experiment and were fed a semisynthetic diet through the gastric cannula and allowed 0.23% (w/v) saline to drink ad lib.

Diets

All unoperated animals were maintained on ground Formulab chow containing 3% triolein. In specific experiments, 3% cholestyramine (Mead Johnson Laboratories, Evansville, Ind.) or 1% cholesterol (Difco Laboratories, Detroit, Mich.) was also added to this ground diet. The operated animals were all fed a semisynthetic diet containing dextrin (Matheson Coleman & Bell, East Rutherford, N.J.), casein (General Biochemicals, Chagrin Falls, Ohio), and water in the proportions of 105:45:175 (by weight) and adjusted to a pH of 7.0 with sodium hydroxide. This liquid diet was delivered to each animal through the indwelling gastric cannula by an infusion pump (B. Braun Apparatebau, Melsungen, W. Germany) at a rate of 1.0 ml/hr.

Tissue preparation and incubation conditions

At the termination of the experiments, each animal was decapitated, and the liver was quickly removed, chilled, and cut into ribbons approximately 2 mm thick. Liver slices 0.8 mm thick were then prepared on a tissue slicer (H. Mickle, Gomshall, England). 300-mg aliquots were placed in 25-ml Erlenmeyer flasks fitted with centerwells and containing 5 ml of oxygenated Krebs bicarbonate buffer (pH 7.4) and the appropriate substrate. Generally, six flasks were run from each animal; in particular experiments they contained either [1-^{14}C]acetate (New England Nuclear, Boston, Mass.) at a concentration of 4

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mM or [1-^{14}C]octanoate (New England Nuclear) at a concentration of 1 mM. Two of the flasks were used in the ketone determinations for zero-time corrections for mass and radioactivity; the remaining flasks were incubated for 90 min at 37°C in a metabolic shaker (Precision Scientific Co., Chicago, Ill.) at 160 oscillations/min. In previous experiments we have shown that this slice thickness and shaker rate give maximal rates of synthesis, that the rates of incorporation of the radiolabeled substrates into various products are linear with respect to time during the 90-min incubation, and that these concentrations of substrates give essentially V_{max} rates (8).

In certain experiments a portion of each liver also was homogenized for direct assay of microsomal HMG CoA reductase activity. This portion of liver was homogenized in a 1:4 (w/v) volume of solution containing 300 mM sucrose, 5 mM dithiothreitol, and 10 mM sodium ethylenediaminetetraacetic acid (pH 7.5) in a Dounce homogenizer with 15 strokes of a loose-fitting pestle. After centrifugation twice for 10 min at 12,100 *g* in a refrigerated centrifuge, the supernate was centrifuged for 60 min at 100,000 *g* at 4°C. The microsomal pellet was then suspended in one-half the original volume of buffer used for homogenization. HMG CoA reductase activity was assayed on aliquots of this suspension as described in detail elsewhere (9). The membrane-bound enzyme (0.1–1.5 mg of protein), diluted to remain in the linear range of the assay, was incubated in 0.5 ml of a solution (pH 7.5) containing 100 mM K_2PO_4, 20 mM glucose-6-phosphate (Sigma Chemical Co., St. Louis, Mo.), 2.5 mM triphosphopyridine nucleotide (Sigma), 0.7 units of glucose-6-phosphate dehydrogenase (Boehringer Mannheim Corp., New York), 6 mM dithiothreitol (Calbiochem, San Diego, Calif.), and 0.174 mM DL-3-hydroxy-3-methyl[3-^{14}C]glutaryl CoA (specific activity, 1.76 Ci/mole). We have established in preliminary experiments that these conditions give maximal enzymatic rates that are linear with respect to time and to protein concentration in the range employed and that the concentration of substrate is 7.5 times the half-maximal value for microsomal HMG CoA reductase. Each assay was performed in triplicate with three different amounts of enzyme protein to confirm linearity with respect to protein concentration, and the results of these triplicate determinations were averaged. After an incubation period of 30 min, the reaction was stopped by the addition of 0.02 ml of 5 N HCl. 3 μmoles of [^{3}H]mevalonolactone (specific activity, 21.8 mCi/mole; New England Nuclear) was added, and the mixture was incubated for 15 min at 37°C to assure lactonization of the mevalonate.

Analytic procedures

The pair of incubation flasks containing [1-^{14}C]acetate was used to determine the rates of incorporation of this precursor into CO_2, long-chain fatty acids, and cholesterol by previously described methods (4). In other experiments, [1-^{14}C]octanoate was used to determine the rates of incorporation of this substrate into CO_2 and cholesterol. In both situations the total radioactivity found in each product was divided by the specific activity of the radiolabeled precursor that was incubated with the slices to yield rates that are expressed as the nanomoles of each respective radiolabeled precursor incorporated into the various products per gram of liver per hour (nmoles·g^{-1}·hr^{-1}). Two other pairs of flasks, one pair incubated at 37°C for 90 min and the other pair kept at 0°C, were utilized to determine the rate of ketone synthesis and the specific activity of the ketone pool when either [1-^{14}C]acetate or [1-^{14}C]octanoate was used as substrate. Acetoacetate and β-hydroxybutyrate were measured by the method of Williamson, Bates, and Krebs (10) as modified by McGarry, Guest, and Foster (11). The rates of synthesis of these two ketones were combined and are reported as the micromoles of total ketones synthesized per gram of liver per hour (μmoles·g^{-1}·hr^{-1}). Total radioactivity incorporated from the substrate into the acetone moiety of acetoacetate was measured by preparation and radioassay of the Denigès salt; the isotope content of the carboxyl group was assayed after liberation of $^{14}CO_2$ by aniline citrate as described by McGarry et al. (11). The data from these determinations were used to calculate the specific activity of the ketone pool as described below. The rates of incorporation of DL-3-hydroxy-3-methyl[3-^{14}C]glutaryl CoA into mevalonic acid were determined by thin-layer chromatography using an internal standard of [^{3}H]mevalonic acid to correct for recovery, as previously described (9, 12). The protein concentrations in the microsomal suspensions were measured by a modification of published methods (13) after precipitation with 5% trichloroacetic acid.

Calculations

As previously described (11), the radioactivity, after subtraction of the zero-time values, in the acetone and carboxyl moieties of the ketones were combined and divided by the mass of total ketones formed during the 90-min incubation. This specific activity value was then compared with the theoretical specific activity that the ketones should have if no endogenous dilution of the acetyl CoA pool occurred. The theoretical specific activity should be twice the specific activity of the radiolabeled substrate when slices were incubated with [1-^{14}C]acetate and half the specific activity of the radiolabeled precursor in experiments using [1-^{14}C]octanoate. In the tables and diagrams, the relative ketone specific activity equals the absolute ketone specific activity divided by the absolute theoretical specific activity times 100.

In specific experiments the rates of incorporation of either [1-^{14}C]acetate or [1-^{14}C]octanoate into various

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products were corrected for intracellular dilution by endogenous acetyl CoA units. In addition, the data from the experiments using [1-^{14}C]octanoate were recalculated to yield incorporation rates in terms of acetyl CoA units, i.e., C_2 units, rather than nanomoles of octanoate. Hence, the corrected C_2 flux from [1-^{14}C]octanoate into CO_2 equals the rate of incorporation of [1-^{14}C]octanoate into CO_2 times 4 times 100 divided by the relative ketone specific activity. The corrected C_2 flux from [1-^{14}C]octanoate into cholesterol is calculated in an identical manner except that the incorporation rate is multiplied by 6 rather than 4 to correct for loss of 33% of the radioactivity as $^{14}CO_2$ during the conversion of [1-^{14}C]acetyl CoA to cholesterol in the reaction generating isopentenylpyrophosphate. Corrected C_2 flux rates of [1-^{14}C]acetate into CO_2 equals the incorporation rate of [1-^{14}C]acetate into CO_2 times 100 divided by the relative ketone specific activity. Corrected C_2 flux rates of [1-^{14}C]acetate into cholesterol cannot be calculated because the specific activity of the extramitochondrial acetyl CoA pool is unknown.

The data on HMG CoA reductase activity were calculated as the nanomoles of mevalonate formed per hour per milligram of microsomal protein. In order to express these data as enzyme activity per gram of liver, this figure was multiplied by 21, the number of milligrams of microsomal protein per gram of liver (14). In these experiments the actual recoveries of microsomal protein ranged from 11.1 to 15.5 mg·g^{-1} liver because the homogenization technique was intentionally gentle in order to avoid disrupting mitochondria and releasing HMG CoA lyase activity. In order also to express these rates in terms of the C_2 flux into mevalonic acid, the data were multiplied by 3, because each mevalonate formed requires 3 C_2 units. All data shown in the tables and figures are mean values ± 1 SEM.

RESULTS

Variation in specific activity of the intramitochondrial acetyl CoA pool when using [1-^{14}C]acetate as substrate

Initial experiments (**Table 1**) were undertaken to determine the magnitude and variability of endogenous dilution of the intramitochondrial acetyl CoA pool when [1-^{14}C]acetate was used as the radiolabeled precursor. The first three groups of animals (groups A, B, and C) were unoperated animals in which the rate of cholesterol synthesis was varied by fasting or by cholesterol feeding. The second set (groups D–G) was operated and restrained animals in which biliary or lymphatic diversion and biliary obstruction were used to alter the rates of synthesis. As anticipated from previous work (3–5), fasting and cholesterol feeding markedly suppressed the rate of [1-^{14}C]acetate incorporation into cholesterol, whereas biliary and lymphatic diversion and biliary obstruction enhanced apparent rates of cholesterogenesis by factors of 2 to 3. The incorporation of [1-^{14}C]acetate into long-chain fatty acids was not significantly suppressed below the appropriate control values except in the case of fasting, where apparent synthesis dropped to 1% of control levels. Fasting and, to a lesser degree, biliary obstruction also suppressed the rate of [1-^{14}C]acetate incorporation into CO_2, but similar suppression was not seen with the other experimental manipulations.

In the operated animals, ketone synthesis differed little among the various groups and ranged from 2.0 ± 0.3 to 3.6 ± 0.9 μmoles·g^{-1}·hr^{-1}. In the unoperated animals, however, fasting enhanced ketone production fourfold over the rates seen in the control or cholesterol-fed animals. The rate of ketone synthesis in the liver is, to a large extent, substrate limited (11, 15, 16) and so reflects the size of the intramitochondrial acetyl CoA pool. Since in these experiments the available exogenous substrate, i.e., radiolabeled acetate, was fixed at a constant concentration, the differences in ketone production rates reflect differences in the intramitochondrial generation of acetyl CoA from endogenous substrates in the various experimental situations. Hence, as can be seen in Table 1, there is generally an inverse relationship between the rate of ketone production and the relative ketone specific activity; the greater the rate of endogenous acetyl CoA generation the higher the rate of ketone synthesis and the lower the relative specific activity of the mitochondrial acetyl CoA pool. As is evident, the degree of dilution varied from approximately twofold in the control animals to nearly fourfold in the fasted animals; i.e., in the latter situation 3 moles of unlabeled acetyl CoA from endogenous sources entered the mitochondrial pool for every mole derived from the exogenous [1-^{14}C]acetate.

Rates of cholesterogenesis determined using [1-^{14}C]octanoate as substrate

In order to determine "true" rates of cholesterol synthesis, i.e., rates of substrate incorporation corrected for endogenous dilution of the precursor pool, additional studies, shown in **Table 2,** were undertaken using [1-^{14}C]octanoate as the substrate. Although absolute rates of incorporation differed, in general, the relative changes in rates of [1-^{14}C]octanoate incorporation into cholesterol and CO_2 caused by different experimental manipulations were similar to those shown in Table 1 ([1-^{14}C]acetate as substrate). Thus, such maneuvers as fasting and cholesterol feeding inhibited substrate incorporation into the two products, whereas cholestyramine feeding, biliary obstruction, and biliary and lymphatic diversion enhanced cholesterogenesis from [1-^{14}C]octanoate.

Octanoate is known to enter the mitochondria rapidly

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TABLE 1. Incorporation of [1-^{14}C]acetate into CO_2, long-chain fatty acids, and cholesterol by liver slices from animals subjected to various experimental manipulations

Experimental Group	n[a]	Animal Weight	Liver Weight	[1-^{14}C]Acetate Incorporation into			Ketone Synthetic Rate	Relative Ketone Specific Activity[b]	Corrected C_2 Flux into CO_2[c]
				CO_2	Fatty Acids	Cholesterol			
		g	*g*	*nmoles · g^{-1} · hr^{-1}*			*μmoles · g^{-1} · hr^{-1}*	*% theoretical*	*nmoles · g^{-1} · hr^{-1}*
Unoperated animals[d]									
A. Control	5	221 ± 13	8.4 ± 0.6	7766 ± 656	1455 ± 394	80 ± 15	1.8 ± 0.1	49 ± 6	15,850 ± 850
B. Fasted 48 hr	5	192 ± 7	5.4 ± 0.2	4119 ± 808	16 ± 3	12 ± 6	9.4 ± 0.5	27 ± 4	15,250 ± 1327
C. Cholesterol-fed	5	205 ± 7	6.9 ± 0.4	7926 ± 354	1365 ± 726	7 ± 3	2.2 ± 0.9	51 ± 4	15,540 ± 2300
Operated and restrained[e]									
D. Control	6	213 ± 6	7.3 ± 0.3	5828 ± 712	421 ± 147	144 ± 13	3.0 ± 0.4	38 ± 13	15,330 ± 2250
E. Biliary fistula	5	198 ± 8	7.1 ± 0.3	6342 ± 472	500 ± 159	390 ± 28	2.0 ± 0.3	49 ± 7	12,940 ± 2565
F. Biliary obstruction	5	191 ± 8	7.0 ± 0.2	4771 ± 620	284 ± 60	268 ± 10	3.6 ± 0.9	34 ± 4	14,030 ± 1423
G. Lymphatic fistula	4	183 ± 12	6.4 ± 0.3	7338 ± 600	635 ± 176	505 ± 39	2.8 ± 0.4	45 ± 7	16,300 ± 2260

[a] Number of animals in each group. Mean values ±1 SEM for all determinations are shown.
[b] Expressed as the percentage of theoretical if no endogenous dilution of the precursor C_2 units occurred.
[c] Rate of incorporation of [1-^{14}C]acetate into CO_2 divided by the relative ketone specific activity times 100.
[d] All animals in this group were maintained in gang cages in continuous light for 1 wk with food and water. Control animals were allowed food up to the time they were killed; fasted animals were allowed water but no food for 48 hr prior to being killed; cholesterol-fed animals were given a 1.0% cholesterol diet for 48 hr prior to being killed.
[e] All animals in this group were conditioned in gang cages in the light for 1 wk prior to use and were then operated on, and a feeding cannula was placed in the stomach. Biliary or lymphatic drainage or biliary obstruction also was performed in specific groups of animals. All groups were fed a semisynthetic diet through the gastric cannula for 48 hr prior to preparation of liver slices.

and to be oxidized to acetyl CoA; hence, it is a better substrate for expanding the intramitochondrial CoA pool than acetate. The difference between acetate and octanoate in this regard is seen in the present study. Liver slices incubated with equivalent amounts of substrate when expressed in moles of C_2 units, i.e., 4 mmoles of C_2 units from both acetate and octanoate, have strikingly different rates of ketone synthesis. Slices from control animals generated ketones at rates of 1.8 to 3.0 μmoles·g^{-1}·hr^{-1} when incubated with [1-^{14}C]acetate but at rates of 7.3 to 8.5 μmoles·g^{-1}·hr^{-1} when incubated with [1-^{14}C]octanoate.

As a consequence of this accelerated intramitochondrial acetyl CoA production, acetyl CoA generated from endogenous sources becomes quantitatively less important, so that the specific activity of the mitochondrial acetyl CoA pool more closely approaches the theoretical value expected from the specific activity of the added exogenous substrate. Thus, relative ketone specific activity varied from 54 ± 3 to 71 ± 3% of the theoretical specific activity in the slices incubated with [1-^{14}C]octanoate while, in the previous experiment, it equaled only 27 ± 4 to 51 ± 4% of the theoretical specific activity when [1-^{14}C]acetate was utilized as the substrate.

In the experiments illustrated in both Tables 1 and 2, fasting produced the greatest endogenous dilution of substrate specific activity, a finding that would be anticipated on the basis of the high rate of fatty acid oxidation and acetyl CoA production known to occur in this metabolic state. In order to define more precisely events occurring in graded degrees of fasting, the studies shown in **Fig. 1** were undertaken. Groups of animals were fed amounts of food varying from 0 to 15 g/24 hr for 48 hr while other groups were allowed food ad lib. Liver slices from each group of animals were then incubated with either [1-^{14}C]acetate or [1-^{14}C]octanoate. All animals had a net weight loss on food intakes of less than 15 g/24 hr; animals receiving 15 g of food/24 hr maintained their weight essentially constant while those allowed ad lib. intake gained approximately 4 g.

As shown in the lower panels in Fig. 1, when acetate was the substrate, ketone synthesis equaled approximately 2–3 μmoles·g^{-1}·hr^{-1} in those animals whose weight remained constant or increased. In contrast, ketone synthesis abruptly increased to 8–9 μmoles·g^{-1}·hr^{-1} in those animals that lost weight during the 48-hr experimental period. When [1-^{14}C]octanoate was the substrate, ketone synthesis increased more gradually, from approximately 7.5 to 15 μmoles·g^{-1}·hr^{-1} in animals subjected to a progressively smaller food intake. Coincidentally with these changes in ketone production, the relative ketone specific activity decreased from 60 to 34% of theoretical and from 80 to 58% of theoretical when [1-^{14}C]acetate and [1-^{14}C]octanoate, respectively, were used as substrates.

Relationship between rates of synthesis of cholesterol determined with [1-^{14}C]octanoate and microsomal HMG CoA reductase activity

In the final experiment, a comparison was made between rates of synthesis of cholesterol determined using [1-^{14}C]octanoate and [1-^{14}C]acetate as substrates and

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TABLE 2. Incorporation of [1-^{14}C]octanoate into CO_2 and cholesterol by liver slices from animals subjected to various experimental manipulations

Experimental Group	n[a]	Animal Weight	Liver Weight	[1-^{14}C]Octanoate Incorporation into CO_2	[1-^{14}C]Octanoate Incorporation into Cholesterol	Ketone Synthetic Rate	Relative Ketone Specific Activity[b]	Corrected C_2 Flux into CO_2[c]	Corrected C_2 Flux into Cholesterol[d]
		g	*g*	*nmoles · g^{-1} · hr^{-1}*		*μmoles · g^{-1} · hr^{-1}*	*% theoretical*	*nmoles · g^{-1} · hr^{-1}*	
Unoperated animals									
A. Control[e]	7	210 ± 8	7.6 ± 0.2	2190 ± 78	49 ± 14	7.3 ± 0.7	64 ± 2	13,690 ± 603	459 ± 127
B. Cycled, light phase[f]	5	207 ± 7	7.7 ± 0.7	2093 ± 83	27 ± 4	6.7 ± 0.3	61 ± 2	13,720 ± 190	265 ± 38
C. Cycled, dark phase[f]	5	212 ± 12	6.8 ± 0.3	2445 ± 19	109 ± 18	7.1 ± 0.5	67 ± 8	14,600 ± 1480	976 ± 276
D. Fasted, 24 hr[e]	4	192 ± 6	4.9 ± 0.3	1639 ± 76	13 ± 4	16.9 ± 0.6	54 ± 3	12,140 ± 750	144 ± 38
E. Fasted, 72 hr[e]	4	172 ± 3	4.2 ± 0.2	1547 ± 58	4 ± 2	15.9 ± 0.6	54 ± 3	11,460 ± 670	44 ± 22
F. Cholestyramine-fed[e]	5	208 ± 6	7.6 ± 0.4	2295 ± 92	154 ± 3	7.9 ± 0.9	62 ± 3	14,800 ± 540	1490 ± 71
G. Cholesterol-fed[e]	4	205 ± 5	7.4 ± 0.4	1960 ± 101	1 ± 1	7.6 ± 0.6	65 ± 3	12,060 ± 950	9 ± 2
Operated and restrained[g]									
H. Control	7	199 ± 7	6.8 ± 0.2	1921 ± 114	38 ± 7	8.5 ± 1.0	68 ± 3	11,300 ± 1020	335 ± 52
I. Biliary Fistula	7	193 ± 5	6.2 ± 0.3	1677 ± 106	148 ± 15	8.7 ± 0.8	65 ± 4	10,320 ± 620	1366 ± 160
J. Biliary Obstruction	6	183 ± 3	7.8 ± 0.3	1463 ± 96	115 ± 13	8.5 ± 1.0	71 ± 2	8,242 ± 590	972 ± 101
K. Lymphatic Fistula	4	173 ± 3	6.1 ± 0.1	1840 ± 42	183 ± 13	8.6 ± 0.4	71 ± 3	10,366 ± 390	1546 ± 143

[a] Number of animals in each group. Mean values ± 1 SEM for all determinations are shown.

[b] Expressed as the percentage of theoretical if no endogenous dilution of the precursor C_2 units occurred.

[c] Calculated by multiplying [1-^{14}C]octanoate incorporation rates into CO_2 by 4, dividing by the relative ketone specific activity, and multiplying by 100.

[d] Calculated by multiplying [1-^{14}C]octanoate incorporation rates into cholesterol by 6, dividing by the relative ketone specific activity, and multiplying by 100.

[e] All animals in this group were maintained in gang cages in light for 1 wk with food and water. Control animals were allowed food up to the time they were killed; fasted animals were allowed water but no food for 24 or 72 hr prior to being killed; cholestyramine-fed animals were given a 3% cholestyramine diet for 72 hr prior to being killed; cholesterol-fed animals were given a 1.0% cholesterol diet for 48 hr prior to being killed.

[f] Animals were cycled for 10 days through alternating 12-hr light and dark cycles and killed at the midpoints of either the light or dark periods.

[g] All animals in this group were conditioned in gang cages in the light for 1 wk prior to use and then operated on, and a feeding cannula was placed in the stomach. Biliary or lymphatic drainage or biliary obstruction also was performed in specific groups of animals. All groups were fed a semisynthetic diet through the gastric cannula for 48 hr prior to preparation of liver slices.

microsomal HMG CoA reductase activity in the same livers. In this study, sterol synthetic rates were varied in different animals by fasting, cholesterol feeding, diurnal light cycling, and feeding cholestyramine. One portion of the liver from each animal was assayed for HMG CoA reductase activity and another portion was incubated with either [1-^{14}C]octanoate or [1-^{14}C]acetate. As shown in the left panel of **Fig. 2,** there was an essentially linear relationship and an excellent correlation (r = 0.96) between the corrected C_2 flux from octanoate into cholesterol (vertical axis) and microsomal HMG CoA reductase activity (horizontal axis) over a range of enzymatic activity that varied nearly 100-fold. In contrast, the correlation between the incorporation rates of acetate into cholesterol and HMG CoA reductase was not as good (r = 0.79) and, in specific manipulations such as fasting, there was marked underestimation of rates of cholesterol synthesis using this radiolabeled substrate.

DISCUSSION

The rate of cholesterol synthesis in the liver is known to be mediated via alterations in activity of the rate-limiting enzyme, HMG CoA reductase (5, 9). Control of this enzyme is complex and still poorly understood. Part of the difficulty arises from the fact that the rate of sterol synthesis is altered by such diverse maneuvers as decreased caloric intake, surgical stress, restraint, qualitative changes in the diet, interruption of the enterohepatic circulation of bile acids, and variations in the enterolymphatic circulation of cholesterol (5).[1] Attempts, therefore, to interpret alterations in HMG CoA reductase activity brought about by a specific experimental maneuver must take cognizance of the many secondary or nonspecific changes that may have been brought about by a particular maneuver. Thus, the ability to measure the rate of ketone synthesis, fatty acid synthesis, CO_2 production, or other metabolic pathways, as well as the rate of cholesterol synthesis, provides important information about the metabolic state of the animal.

However, our recent observations that there is significant intracellular dilution of the precursor pool when radiolabeled acetate is used as the substrate and, further, that the acetyl CoA pools in the mitochondria and cytosol are not in equilibrium raise certain problems with the use of this radiolabeled substrate (8). As is apparent in Table

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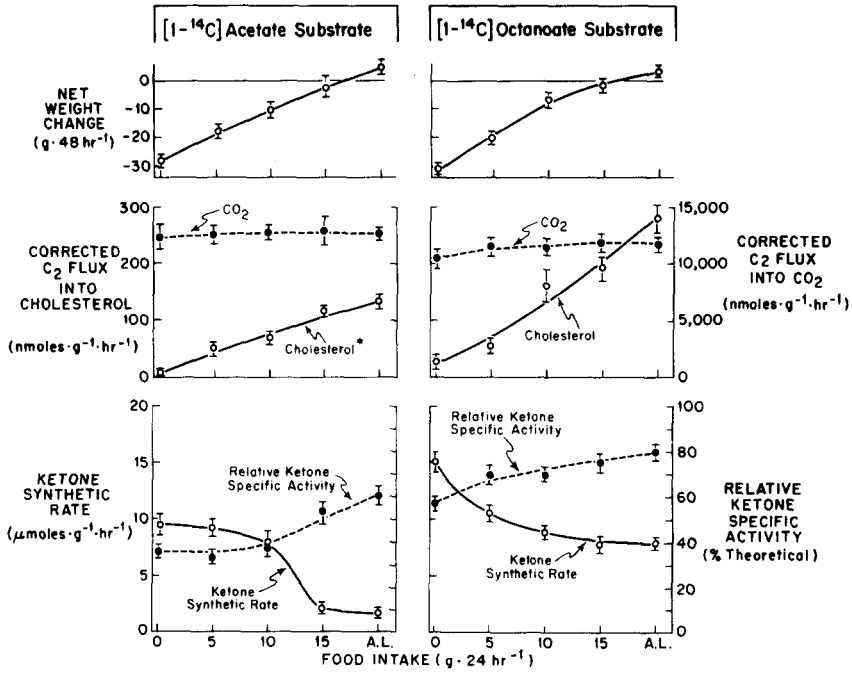

Fig. 1. Effect of varying food intake on the rates of CO_2, cholesterol, and ketone synthesis and on the relative ketone specific activity. All animals were maintained in continuous light for 10 days prior to use. They were then placed in individual metabolic cages and for 2 days were fed ground rat chow in amounts of 0, 5, 10, or 15 g/24 hr or were allowed ad lib. (A.L.) access to food. Net weight changes during the 48-hr experimental period are shown in the two upper panels. At the end of this time the animals were killed, and liver slices were incubated with either [1-^{14}C]acetate or [1-^{14}C]octanoate. Corrected C_2 flux rates from [1-^{14}C]acetate into CO_2 and from [1-^{14}C]octanoate into CO_2 and cholesterol are shown; the C_2 flux from [1-^{14}C]acetate into cholesterol (*) is not corrected for endogenous dilution because the specific activity of the extramitochondrial acetyl CoA pool is unknown in these experiments. Mean values ± 1 SEM are shown for four animals in each group.

1, the specific activity of the intramitochondrial acetyl CoA pool, as evidenced by the relative ketone specific activity, varies widely depending upon the metabolic state of the animal. Since CO_2 is generated from this pool, it is possible to correct the flux of [1-^{14}C]acetate into this product for the variation in precursor pool specific activity; for example, the rate of incorporation of [1-^{14}C]acetate into CO_2 in fasted animals, 4119 ± 808 nmoles·g^{-1}·hr^{-1}, is significantly lower than the rate of 7766 ± 656 nmoles·g^{-1}·hr^{-1} found in fed control animals. Yet, after correction for differences in the intramitochondrial precursor pool specific activity, as shown in the last column of Table 1, the rates of C_2 flux into CO_2 equal 15,250 ± 1327 and 15,850 ± 850 nmoles·g^{-1}·hr^{-1}, respectively, in the fasted and control animals. Thus, endogenous dilution of the acetyl CoA pool resulted in artifactually low rates in both experimental groups and in an artifactual difference in CO_2 production between the fasted and control animals.

Similar differences undoubtedly exist in the specific activity of the cytosolic acetyl CoA pool that gives rise to cholesterol. However, the specific activity of the intramitochondrial pool cannot be used to correct the flux rates of [1-^{14}C]acetate into cholesterol because in slices incubated with this precursor these two pools are probably not in equilibrium. Thus, it is impossible to determine from the data in Table 1 either the absolute rate of cholesterol synthesis or the magnitude of the changes in synthesis that are produced by a given experimental manipulation. It should be emphasized that this problem is not peculiar to the assessment of cholesterol synthetic activity; in any whole cell preparation the flux of radiolabeled carbon from any precursor through the cytosolic acetyl CoA pool into any product presumably is subject to the same unknown and variable dilution of specific activity. Hence, there may be artifacts of considerable magnitude in data generated by this means.

The use of octanoate, therefore, has two important advantages. First, because of the high rate of acetyl CoA generation from this exogenous substrate, acetyl CoA production from intracellular substrates becomes relatively less important and the specific activity of the acetyl CoA pool more closely approaches the theoretical value anticipated if no endogenous dilution occurred. Second, because in tissue slices incubated with octanoate the intramitochondrial pool of acetyl CoA presumably is the major source of extramitochondrial C_2 units, the relative specific activity of the ketone bodies can be used to correct the ob-

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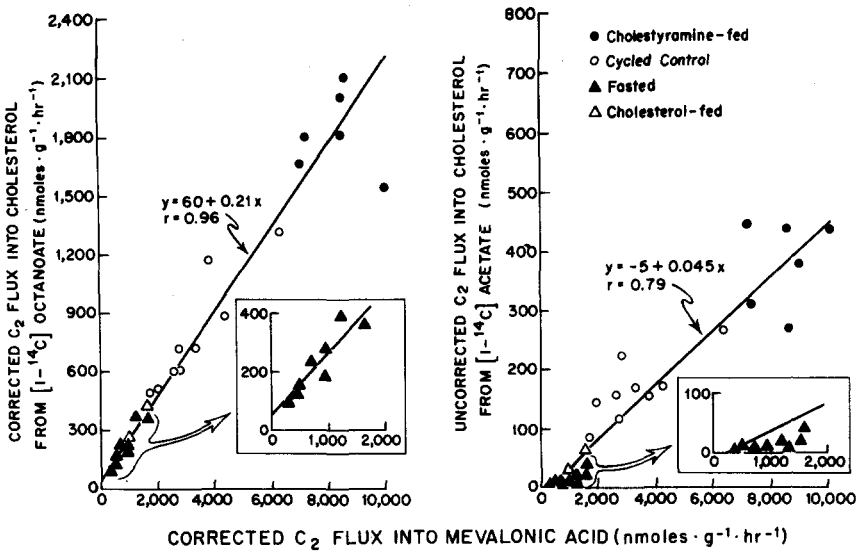

Fig. 2. Correlation of the corrected C_2 flux from [1-^{14}C]octanoate and of the uncorrected C_2 flux from [1-^{14}C]-acetate into cholesterol with HMG CoA reductase activity in the same livers. In order to have a broad spectrum of synthetic activity, livers from fasted, cholesterol-fed, light-cycled control, and cholestyramine-fed animals were assayed for HMG CoA reductase activity and for the rates of cholesterogenesis from [1-^{14}C]octanoate and [1-^{14}C]-acetate. The regression curves were fitted by the method of least squares. Each point represents a single animal.

served flux rate of the radiolabeled precursor into cholesterol for the flow of unlabeled carbon through this pathway.

Using this procedure, absolute rates of incorporation of C_2 units into cholesterol are shown for a variety of physiological situations in Table 2. As is apparent in the last two columns, with the exception of prolonged fasting, the rates of C_2 flux into CO_2 are essentially the same in all experimental situations, whereas the rate of cholesterogenesis varies widely. Control animals maintained in constant light incorporated C_2 units into sterols at a rate of 459 ± 127 nmoles·g^{-1}·hr^{-1} while this rate varied from a low of 265 ± 38 to a high of 976 ± 276 during the diurnal rhythm induced by cyclic lighting. These values are much higher than those obtained previously in studies using radiolabeled acetate as substrate under somewhat different incubation conditions where incorporation rates of approximately 90 nmoles·g^{-1}·hr^{-1} were reported in comparable control animals (5). Similarly, animals with interruption of the enterohepatic circulation of bile acids showed incorporation rates of acetate into cholesterol of approximately 300 nmoles·g^{-1}·hr^{-1} (5), whereas C_2 flux rates of 1366 to 1490 nmoles·g^{-1}·hr^{-1} were calculated in the present study.

Not only does endogenous dilution of substrate specific activity cause erroneously low estimates of rates of cholesterogenesis, but variation in the degree of dilution in different experimental situations also introduces errors into the determination of relative rates of sterol synthesis. This can best be seen by comparing the relative rates of cholesterol synthesis from acetate (Table 1) and from octanoate (Table 2). For example, using [1-^{14}C]acetate as substrate, biliary diversion, biliary obstruction, and lymphatic diversion enhance the apparent rates of synthesis by 270%, 186%, and 350%, respectively. In contrast, using [1-^{14}C]octanoate these same manipulations increased synthesis by 407%, 290%, and 461%, respectively. These differences in relative rates of synthesis presumably reflect alterations in the specific activity of the cytosolic acetyl CoA pool that escape detection when [1-^{14}C]acetate is the precursor.

These data, then, demonstrate that significant and variable intracellular dilution of the cytosolic acetyl CoA pool occurs in liver slices that results in gross underestimation of absolute rates of cholesterol synthesis as well as in the introduction of errors in the determination of relative incorporation rates. These errors can be minimized by using radiolabeled octanoate as substrate and correcting for intracellular dilution of the specific activity of the precursor pool. Other methods can yield similar corrections. For example, use of tritiated water as substrate should give accurate relative rates of cholesterogenesis because changes in the metabolic state of the hepatic cell do not alter the specific activity of intracellular water. However, this method will not yield absolute rates of cholesterol synthesis unless one also knows the ratio that defines the number of ^{3}H atoms incorporated into cholesterol per carbon atom. The value of this ratio has been experimentally determined by Brunengraber et al. (17) and applied to studies of cholesterogenesis in the perfused liver. It is of interest that these authors obtain a rate of C_2 incorporation into cholesterol in the perfused organ of 1.40 μmoles·g^{-1}·hr^{-1} in fed control animals compared with rates in liver slices from control animals in the present study that were 0.27 to 0.98

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μmoles·g^{-1}·hr^{-1}, depending upon the phase of the light cycle.

Finally, it is of considerable importance to compare in the same livers the rates of cholesterogenesis determined in slices using either [1-^{14}C]acetate or [1-^{14}C]octanoate with microsomal HMG CoA reductase activity. As seen in the left panel of Fig. 2, there is an excellent correlation between the corrected C_2 flux from [1-^{14}C]octanoate into cholesterol and the rate of HMG CoA reductase. When [1-^{14}C]acetate is used as the precursor, however, the correlation is much poorer. In addition, in manipulations such as fasting, where there is augmented fatty acid oxidation and much greater dilution of the cytosolic acetyl CoA pool, the acetate incorporation rate may underestimate HMG CoA reductase activity by several hundred percent.

The fact that the rate of cholesterol synthesis measured with [1-^{14}C]octanoate is proportional to the activity of HMG CoA reductase over a wide range of synthetic rates suggests strongly that, under each of the physiological manipulations employed, HMG CoA reductase activity was rate limiting in the overall synthesis of cholesterol from C_2 precursors. Nevertheless, under the optimal conditions employed in the enzyme assay, the absolute rate of HMG CoA conversion to mevalonate was threefold higher than the rate of C_2 incorporation into cholesterol by slices taken from the same livers. This indicates that, under the in vitro conditions employed in the slice experiments, the enzyme was functioning at about one-third of its maximal capacity. It may be that, in the intact cell, enzyme activity is limited by factors such as the availability of TPNH, nonoptimal pH, the effects of intracellular compartmentalization on the microsome, or the availability of sulfhydryl reducing agents on which enzyme activity strongly depends (9). However, the observation that the rate of HMG CoA reductase activity was higher than the observed rate of C_2 unit incorporation into cholesterol with every physiological manipulation suggests that the factors that limit intracellular HMG CoA reductase activity do not vary with the physiological state of the animal. Hence, the flux of C_2 units into cholesterol in tissue slices precisely mirrors the relative activity of HMG CoA reductase activity in all physiological states tested.

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