Limitations of Acetate as a Substrate for Measuring Cholesterol Synthesis in Liver*

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JOHN M. DIETSCHEY and J. DENIS McGARRY

From the Departments of Internal Medicine and Biochemistry, The University of Texas Southwestern Medical School at Dallas, Dallas, Texas 75235

SUMMARY

In studies designed to measure rates of hepatic cholesterogenesis, rat liver slices were incubated with [14C]acetate in the presence of unlabeled octanoate and with [14C]octanoate in the presence of unlabeled acetate to assess the degree of intracellular dilution and compartmentalization of the acetyl-CoA pools. It was observed that when increasing concentrations of [14C]acetate were used, the incorporation rate of this substrate into cholesterol became constant under circumstances where the specific activity of the ketone bodies continued to rise. Second, the addition of unlabeled acetate reduced the flow of radiolabeled C$_2$ units from [14C]octanoate into cholesterol but not into ketone bodies or CO$_2$. Third, use of the specific activity of the ketone bodies was shown to yield an appropriate correction for the flux of [14C]octanoate into cholesterol: in contrast, this correction was shown to be inappropriate in experiments in which cholesterogenesis was measured using radiolabeled acetate. Taken together, these findings indicate that [14C]acetate gives rise to a cytosolic pool of acetyl-CoA that is used for cholesterol synthesis but is not in isotopic equilibrium with the mitochondrial pool of acetyl-CoA used for ketogenesis.

From a combination of experiments using acetate and octanoate, singly and together and alternately labeled, the apparent and absolute fluxes of C$_2$ units through both pools were measured. Maximum rates of sterol synthesis were obtained in the presence of octanoate. In contrast, with [14C]acetate as substrate the flux of labeled C$_2$ units into cholesterol was only 47% of maximum while total C$_2$ flux was 75% of that observed with octanoate. Thus, in addition to the errors associated with intracellular dilution when [14C]acetate is used, a further limitation to the use of this substrate appears to be the presence of a partially rate-limiting step in its metabolism prior to hydroxymethylglutaryl-CoA reductase.

The most commonly employed technique for studying rates of hepatic cholesterogenesis has involved the incubation of liver slices with radiolabeled acetate and the subsequent calculation of the amount of this substrate incorporated into cholesterol (1). While this approach has generally yielded valuable qualitative information on the effects of various experimental manipulations on hepatic sterol synthesis, its usefulness in assessing absolute rates of cholesterogenesis is based on a number of assumptions the validity of which has never been firmly established. Thus, it has been assumed that the rate of penetration of the labeled acetate into the cell and its activation to acetyl-CoA is not rate-limiting and that the rate of the latter process greatly exceeds the endogenous rate of acetyl-CoA production so that the specific activity of the C$_2$ pool feeding the enzymes of cholesterogenesis can be considered essentially equal to that of the added substrate. If, however, significant endogenous dilution of the labeled precursor is suspected it might be supposed, as reported recently (2), that true rates of sterol synthesis could be calculated by correcting the observed rate of labeled acetate incorporated into cholesterol for alterations in the specific activity of the acetyl-CoA pool as determined, for example, by measurements of the specific activity of the ketone bodies formed during the incubation. Implicit in this procedure is the further assumption that with acetate as the added substrate the acetyl-CoA used for both ketogenesis and cholesterol synthesis derives either from one pool or from separate pools that are in isotopic equilibrium with one another.

A number of considerations, however, suggest that these assumptions might not be entirely justified. For example, while acetate serves as a good substrate for processes such as fatty acid synthesis and cholesterogenesis, we have been struck by its low capacity to support ketone body production either in rat liver slices or in the intact perfused organ (3, 4).$^1$ Since ketogenesis is known to be primarily an intramitochondrial event (5, 6) it seemed likely that acetate is unable to generate intramitochondrial acetyl-CoA at a rate sufficient to maintain high levels of ketogenesis. Of further interest is the suggestion that despite the identical chemical nature of the initial reactions involved in both ketogenesis and cholesterogenesis, i.e. the conversion of acetyl-CoA into HMG-CoA$^2$ through the sequential action of $\beta$-ketothiolase and HMG-CoA synthase, it is probable that the same pools of intermediates are not utilized in the two biosynthetic pathways (4, 7, 8). The suggestion has been made, therefore, that the entire sequence of reactions leading from acetyl-CoA

$^1$ Unpublished observations

$^2$ The abbreviation used is: HMG-, hydroxymethylglutaryl-
to cholesterol might take place in the extramitochondrial compartment of the cell. Taken together with the fact that acetate can be activated to its coenzyme A derivative by an enzyme present in the cytosolic fraction of hepatocytes (9), these considerations could have two important consequences with regard to calculations of rates of cholesterol synthesis with radiolabeled acetate as the substrate. The first is that the extent of incorporation of the labeled acetate into sterols might grossly underestimate total C3 flux through this pathway. Second, if the mitochondrial pool of HMG-CoA used in acetoacetate formation is not in isotopic equilibrium with that utilized for mevalonic acid synthesis on the microsomes then changes in the specific activity of the ketone bodies formed could not be used to correct the apparent C3 flux into cholesterol for the incorporation of endogenously produced acetyl-CoA.

The present investigations were designed to examine these questions. In the studies described below we have compared rates of cholesterol synthesis in rat liver slices using [1-14C]acetate and [1-14C]octanoate as precursors. The former presumably would generate labeled acetyl-CoA in the cytosolic compartment while the latter would generate acetyl-CoA in the mitochondrion. By incubating slices with both substrates simultaneously under conditions where one or the other carried the 14C label we sought to determine whether the ketone bodies and cholesterol are derived from the same or from separate pools of acetyl-CoA. The data reveal serious limitations in the use of acetate in this type of study and fully support the concept that the entire cholesterol biosynthetic pathway from acetyl-CoA takes place in the extramitochondrial compartment of the liver cell.

**EXPERIMENTAL PROCEDURE**

**Animals**—Sprague-Dawley-derived female rats (230 to 280 g; Simonsen Laboratories, Gilroy, Calif.) were used in these studies. All animals were maintained for at least 2 weeks on Formulab chow (Ralston Purina Co., St. Louis, Mo.) and water ad libitum prior to use. In certain experiments, as designated under “Results and Discussion,” animals were exposed to alternating 12-hour cycles of light and dark by placing the rats in light-tight chambers with forced air ventilation. Lighting in these chambers was controlled by timers and changed at 03:00 and 15:00 hours. By this means, animals were available at 09:00 hours that were in either the mid-dark or mid-light period of the cycle, i.e. 6 hours after the onset of the dark or light cycle, respectively.

**Tissue Preparation and Incubation Conditions**—Animals were decapitated and the livers were quickly removed, chilled, and cut into ribbons approximately 2 mm thick. Liver slices were then prepared on a tissue slicer (H. Mickle, Gomshall, England) and varying amounts were placed in 25-ml Erlenmeyer flasks fitted with center wells 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 [1-14C]- or [2-14C]acetate (New England Nuclear, Boston, Mass.) or [1-14C]octanoate (New England Nuclear, Boston, Mass.). Two of the flasks were used for zero time corrections of mass and radioactivity in the ketone determinations while the remaining flasks were incubated at 37° in a metabolic shaker (Precision Scientific Corp., Chicago, Ill.). Specific data on slice thicknesses, shaker rates, times of incubation, and substrate concentrations are given in the initial experiments reported under “Results and Discussion.”

**Analytical Procedures and Calculations**—One pair of the flasks incubated at 37° was used to determine the rates of incorporation of radiolabeled substrates into CO2 and cholesterol by previously described methods (10). In both situations the total radioactivity found in each product was divided by the specific activity of the radiolabeled precursor to yield rates that are expressed as the nanomoles of labeled precursor incorporated into the two products per g of liver per hour (nanomoles g-1 hour-1). The remaining pair of flasks was utilized to determine the rate of synthesis of the ketone bodies and their specific activity. Acetoacetate and β-hydroxybutyrate were measured by the method of Williamson et al. (11) as modified by McGarry et al. (12). The production rates of each were combined and are reported as the micromoles of total ketones synthesized per g of liver per hour (micromoles g-1 hour-1). Total radioactivity incorporated into carbon atoms 1 and 3 of acetoacetate and β-hydroxybutyrate was measured as previously described (19).

The observed value for the specific activity of the ketones was then compared with the theoretical value expected if no endogenous dilution of the acetyl-CoA pool occurred. The latter should be twice the specific activity of the radiolabeled substrate when slices were incubated with [2-14C]- or [1-14C]acetate and half the specific activity of the radiolabeled precursor in experiments using [1-14C]octanoate. In the table and diagram the term “relative ketone specific activity” equals the absolute ketone specific activity divided by the theoretical specific activity times 100.

In specific experiments the rates of incorporation of either [1-14C]acetate or [1-14C]octanoate into cholesterol were corrected for intracellular dilution by endogenous acetyl-CoA units. In addition, the data from experiments with [1-14C]octanoate as substrate were used to calculate incorporation rates in terms of acetyl-CoA units, i.e. C3 units, rather than nanomoles of octanoate. Hence, the corrected C3 flux from [1-14C]octanoate into cholesterol equals the rate of incorporation of [1-14C]octanoate into cholesterol times 4 times 1.5 times 100 divided by the relative ketone specific activity. The factor of 4 converts the incorporation rates of octanoate, expressed as nanomoles g-1 hour-1, to incorporation rates of C3 units, also expressed as nanomoles g-1 hour-1. The additional factor of 1.5 corrects for loss of 33% of the radioactivity of 14CO2 during the conversion of [1-14C]acetyl-CoA into cholesterol.

**RESULTS AND DISCUSSION**

Initial experiments were undertaken to determine the conditions of incubation of liver slices that gave maximum substrate incorporation rates into cholesterol. As shown in Fig. 1, the incorporation of [2-14C]acetate into CO2 and cholesterol increased as the rate of agitation of the metabolic shaker was increased; the incorporation rates essentially plateaued at shaker rates of approximately 120 to 200 oscillations per min. Since a rate of 200 oscillations per min occasionally caused the incubation fluid to spill into the center well of the flasks, all subsequent incubations were carried out at 160 oscillations per min. Incorporation rates also increased with slice thickness up to approximately 0.8 mm above which they declined. As shown in the final two panels of Fig. 1 when using tissue slices 0.8 mm in thickness and a shaker rate of 160 oscillations per min, the incorporation of [2-14C]acetate into CO2 and cholesterol was linear with respect to time up to 100 min of incubation and with respect to the weight of slices added to each incubation flask up to 400 mg. Essentially identical results were obtained when [1-14C]octanoate was used as substrate; hence, in most subsequent experiments using either acetate or octanoate as substrate, 300 mg of liver slices, 0.8 mm thick, were incubated for 90 min at a shaker rate of 160 oscillations per min.
In these studies the specific activity of the ketone bodies formed during the period of incubation was used to assess indirectly the specific activity of the intramitochondrial acetyl-CoA pool so as to provide a means for correcting the flux of radiolabeled C3 units through this pool for dilution by endogenously produced, unlabeled C3 units. In order to utilize this method of correction, however, it was imperative that the specific activity of the ketones measured at the end of the experiment reflected the specific activity of the ketones throughout the incubation period. If this were not the case, i.e. if the specific activity of the intramitochondrial acetyl-CoA pool were changing constantly during the incubation, then corrections based on the final ketone specific activity might be in serious error. In order to evaluate this point when either radiolabeled acetate or octanoate was the substrate, the experiments in Fig. 2 were undertaken. As is apparent, the rate of incorporation of both substrates into cholesterol, on the rate of ketone synthesis, and on the relative specific activity of the ketone pool. The conditions of incubation were identical with those described in Fig. 2 except that the concentration of the two substrates was varied and the time of incubation was 90 min. Mean values ± 1 S.E. are shown for six different pools of liver slices.

FIG. 1. The relationship of metabolic shaker speed, slice thickness, time of incubation, and tissue weight to the rates of incorporation of [2-14C]acetate into CO2 and cholesterol. In all studies the 5 ml of incubation medium in each flask contained 20 μmoles of sodium acetate and 2 μCi of [2-14C]acetate. The shaker speed was 100 oscillations per min in Experiments B, C, and D. Liver slice thickness equaled 0.8 mm in Experiments A, C, and D. The incubation time lasted 90 min in Experiments A, B, and D. Three hundred milligrams of liver slices were used in all studies except Experiment D. Mean values ± 1 S.E. are shown for six separate pools of liver slices.

FIG. 2. Time course for the incorporation of [2-14C]acetate and [1-14C]octanoate into cholesterol, for ketone synthesis, and for the relative specific activity of the ketone pool. The 5 ml of incubation medium in each flask contained either 20 μmoles of sodium acetate and 2 μCi of [2-14C]acetate or 5 μmoles of sodium octanoate and 1 μCi of [1-14C]octanoate. Three hundred milligrams of liver slices, 0.8 mm thick, were used and incubations were carried out at 100 oscillations per min. Mean values ± 1 S.E. are shown for six different pools of liver slices.

FIG. 3. The effect of varying substrate concentration on the rate of incorporation of [2-14C]acetate and [1-14C]octanoate into cholesterol, on the rate of ketone synthesis, and on the relative specific activity of the ketone pool. The conditions of incubation were identical with those described in Fig. 2 except that the concentration of the two substrates was varied and the time of incubation was 90 min. Mean values ± 1 S.E. are shown for six different pools of liver slices.
RELATIVE KETONE '0° & --,-g--~-+-~

values f 1 S.E. are given for four different pools of slices. flasks to give a final concentration ranging from 0 to 5 mM. Mean of sodium octanoate, and 1 &i of [l-14C]octanoate. In addition, lesterol and on the specific activity of the ketone pool. The incu-

activit'y and on the rate of 5 mM had only a minimal effect on the relative ketone specific activity and on the concentration of substrate ketone synthesis from octanoate was manyfold greater than from acetate regardless of whether the concentration of available substrate. In addition, it is seen that at any concentration of substrate ketone synthesis from octanoate was manyfold greater than from acetate regardless of whether the concentration of the fatty acid was expressed as millimolar with respect to octanoate or with respect to available C2 units. The two radiolabeled precursors also behaved differently with respect to the specific activity of the ketones achieved at different substrate concentrations. As the concentration of octanoate was raised, the specific activity of ketones approached 90% of the theoretical value indicating that intramitochondrial sources of unlabeled C2 units were essentially swamped out. In contrast, the specific activity of the ketones continued to increase as the concentration of radiolabeled acetate was raised from 0.5 to 12 mM, and even at the highest concentration of acetate, equaled only 50% of the theoretical specific activity.

The fact that the incorporation of [2-14C]acetate into cholesterol reached a constant value under circumstances where the specific activity of the ketone bodies was continually rising suggested that the acetyl-CoA pool from which cholesterol is derived is not in equilibrium with that feeding the pathway of ketogenesis. A second type of experiment, shown in Fig. 4, also supported this conclusion. Here it is seen that the addition of acetate up to 5 mM had only a minimal effect on the relative ketone specific activity and on the rate of incorporation of the radiolabeled substrate into cholesterol to a degree that was inversely proportional to the amount of unlabeled acetate added to the incubation solution. Again, this result indicated that exogenous acetate entered into and significantly diluted the acetyl-CoA pool feeding the enzymes of cholesterol biosynthesis but did not appreciably alter the intramitochondrial pool from which ketones are derived.

Thus, to summarize, these experiments show that when [14C]-

acetate is used as substrate, not only is there marked intracellular dilution of the radiolabeled precursor, but in addition, the acetyl-CoA pools giving rise to ketones and CO2 on one hand and to cholesterol on the other are not in isotopic equilibrium. These conclusions are based on three lines of evidence. First, under experimental conditions where the incorporation of radiolabeled acetate into cholesterol becomes constant and independent of further increases in the concentration of substrate in the incubation media, the specific activity of the acetyl-CoA pool giving rise to steroids must have attained an essentially constant value. However, as shown in Fig. 3, the incorporation of [2-14C]acetate into cholesterol became constant under circumstances where the specific activity of the ketones and, hence, of the intramitochondrial acetyl-CoA pool, continued to increase. Second, if choles terogenesis were taking place from a pool of acetyl-CoA in equilibrium with the intramitochondrial pool then it should be possible to correct the rates of radiolabeled acetate incorporation into cholesterol for intracellular dilution using the specific activity of ketones (13). Such calculations can be made from the data presented in Fig. 3. When acetate was used as substrate the specific activity of the intramitochondrial acetyl-CoA pool can appropriately be used to correct the flux rates since this substrate necessarily must pass through the mitochondrial acetyl-CoA pool before entering the cytosol to be incorporated into cholesterol. Hence, such calculations yield corrected flux rates that are somewhat higher than the experimentally determined values but which still show an essentially hyperbolic relationship to substrate concentration. In contrast, corrected flux rates from [14C]acetate show, paradoxically, a marked fall in value as the substrate concentration is increased. There are two possible explanations for this result: either high acetate concentrations in the medium may actually inhibit the synthesis of cholesterol from endogenous substrate or the specific activity of the intramitochondrial acetyl-CoA pool on which these corrections are based do not reflect the specific activity of the acetyl-CoA pool giving rise to cholesterol in slices incubated with acetate. As is evident from the data calculated in Table I, high acetate concentrations do not inhibit choles terogenesis from endogenous substrate; thus, the most likely explanation for these findings is that radiolabeled acetate is incorporated into cholesterol after passage through an acetyl-CoA pool, presumably in the extramitochondrial compartment, that is not in equilibrium with the mitochondrial pool. Third, if acetate enters into a common intracellular acetyl-CoA pool that feeds both cytosolic and intramitochondrial pathways then exogenous, unlabeled acetate should dilute equally the flux of radiolabeled C2 units into the tricarboxylic cycle and into the choles terogenic pathways. As shown in Fig. 4, this was not the case.

Taken together these observations are entirely consistent with the concept that the acetyl-CoA pool within the mitochondrion that gives rise to CO2 and ketones is functionally separate from and not in equilibrium with the acetyl-CoA pool in the cytosol from which cholesterol is synthesized. This conclusion, it should be emphasized, invalidates the use of the ketone specific activity to correct rates of synthesis of cholesterol using radiolabeled acetate as has been attempted by Barth et al. (2).

Fig. 3. It is apparent that the incorporation of [2-%]acetate from which cholesterol is synthesized. This conclusion, it should be emphasized, invalidates the use of the ketone specific activity to correct rates of synthesis of cholesterol using radiolabeled acetate as has been attempted by Barth et al. (2).

Fig. 4. The effect of varying concentrations of unlabeled acetate on the incorporation of_t-14C)octanoate into CO2 and cholesterol and on the specific activity of the ketone pool. The incubation flasks contained 5 ml of Krebe bicarbonate buffer, 5 μmoles of sodium octanoate, and 1 μCi of (1-14C)octanoate. In addition, varying amounts of unlabeled sodium acetate were added to the flasks to give a final concentration ranging from 0 to 5 mM. Mean values ± 1 S.E. are given for different four pools of slices.
TABLE I

Calculation of flux of C₃ units from substrate into cholesterol in dark- and light-adapted animals

Common pools of liver slices from animals subjected to light cycling for 2 weeks and killed in either the mid-dark or mid-light phase were incubated with [1-¹⁴C]acetate in the presence and absence of unlabeled octanoate and with [1-¹⁴C]octanoate in the presence and absence of unlabeled acetate. The asterisk (*) indicates which compound was labeled in a particular experiment. The data in Column D represent the specific activity of the total ketone divided by the theoretical specific activity expected if no dilution by endogenous C₃ units occurred, times 100. In Column E the data from Column B have been multiplied by 4 in experiments where [1-¹⁴C]octanoate was used. In Column F the incorporation rates of labeled C₃ units have been multiplied by 100 and divided by the relative specific activity values of the ketones (Column D) where [1-¹⁴C]octanoate was the substrate. Finally, all incorporation rates were multiplied by 1.5 (Column G) to correct for loss of 33% of the label during the conversion of ¹⁴C-labeled substrate into cholesterol. Mean values ± 1 S.E. for six determinations are shown.

<table>
<thead>
<tr>
<th>A. Concentration of substrate(s)</th>
<th>B. Incorporation of ¹⁴C-labeled substrate into cholesterol</th>
<th>C. Total ketone synthesis rate</th>
<th>D. Relative ketone specific activity</th>
<th>E. Labeled C₃ units → cholesterol</th>
<th>F. E X 100/D</th>
<th>G. Total C₃ units → cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>Octanoate</td>
<td>μmoles g⁻¹ hr⁻¹</td>
<td>μmoles g⁻¹ hr⁻¹</td>
<td>% theoretical</td>
<td>μmoles g⁻¹ hr⁻¹</td>
<td>μmoles g⁻¹ hr⁻¹</td>
</tr>
<tr>
<td>Dark-adapted animals</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0*</td>
<td>0</td>
<td>99.9 ± 4.5</td>
<td>2.0 ± 0.1</td>
<td>58.0 ± 4.0</td>
<td>99.9 ± 4.5</td>
<td>150.0 ± 6.7</td>
</tr>
<tr>
<td>4.0*</td>
<td>1.0</td>
<td>65.7 ± 6.4</td>
<td>7.9 ± 0.6</td>
<td>13.5 ± 0.2</td>
<td>65.7 ± 6.4</td>
<td>98.5 ± 9.6</td>
</tr>
<tr>
<td>0</td>
<td>1.0*</td>
<td>51.5 ± 3.7</td>
<td>7.0 ± 0.4</td>
<td>88.5 ± 0.3</td>
<td>206.0 ± 14.8</td>
<td>349.1 ± 25.1</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0*</td>
<td>30.3 ± 2.3</td>
<td>7.0 ± 0.1</td>
<td>76.5 ± 1.2</td>
<td>121.3 ± 0.2</td>
<td>227.6 ± 18.0</td>
</tr>
<tr>
<td>0</td>
<td>0.01*</td>
<td>0.732 ± 0.051</td>
<td>1.6 ± 0.6</td>
<td>3.3 ± 0.6</td>
<td>2.9 ± 0.2</td>
<td>87.9 ± 20.7</td>
</tr>
<tr>
<td>4.0</td>
<td>0.01*</td>
<td>0.435 ± 0.071</td>
<td>1.8 ± 0.2</td>
<td>2.7 ± 0.1</td>
<td>1.7 ± 0.3</td>
<td>62.9 ± 11.1</td>
</tr>
<tr>
<td>Light-adapted animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0*</td>
<td>0</td>
<td>30.3 ± 2.8</td>
<td>1.5 ± 0.1</td>
<td>58.5 ± 0.3</td>
<td>30.3 ± 2.8</td>
<td>45.5 ± 4.3</td>
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<tr>
<td>4.0*</td>
<td>1.0</td>
<td>19.2 ± 1.9</td>
<td>7.2 ± 0.4</td>
<td>19.2 ± 1.0</td>
<td>19.2 ± 1.0</td>
<td>28.8 ± 2.8</td>
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<tr>
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<td>6.5 ± 0.5</td>
<td>87.3 ± 4.2</td>
<td>60.8 ± 5.2</td>
<td>104.5 ± 8.9</td>
</tr>
<tr>
<td>4.0</td>
<td>1.0*</td>
<td>10.8 ± 0.8</td>
<td>7.1 ± 0.2</td>
<td>91.2 ± 3.2</td>
<td>43.2 ± 3.2</td>
<td>74.7 ± 3.5</td>
</tr>
<tr>
<td>0</td>
<td>0.01*</td>
<td>0.183 ± 0.014</td>
<td>1.5 ± 0.2</td>
<td>6.0 ± 1.5</td>
<td>0.732 ± 0.056</td>
<td>12.2 ± 0.9</td>
</tr>
<tr>
<td>4.0</td>
<td>0.01*</td>
<td>0.098 ± 0.012</td>
<td>1.5 ± 0.1</td>
<td>4.3 ± 0.4</td>
<td>0.392 ± 0.048</td>
<td>9.1 ± 1.1</td>
</tr>
</tbody>
</table>

In the latter two experiments, the addition of octanoate at a concentration of 1 mM greatly accelerated the generation of intramitochondrial acetyl-CoA as evidenced by the high rate of ketone synthesis. Hence, the observed flux rates of [1-¹⁴C]octanoate into cholesterol represented measurements made under driven conditions. In order to estimate the flux of C₃ units from the mitochondrial pool into cholesterol under circumstances where the pool was not expanded, slices also were incubated with trace amounts, i.e. 0.01 mM, of [1-¹⁴C]octanoate in the presence and absence of unlabeled acetate (Lines 5 and 9 of Table I). In the absence of acetate an incorporation rate of 0.732 ± 0.051 nmole g⁻¹ hour⁻¹ was observed. Ketone synthesis proceeded slowly at a rate of 1.6 ± 0.6 μmoles g⁻¹ hour⁻¹ and the relative specific activity of the ketones was only 3.3 ± 0.6% of theoretical. Again, the addition of unlabeled acetate decreased the incorporation rate of octanoate into cholesterol to 0.435 ± 0.071 nmole g⁻¹ hour⁻¹ but did not significantly reduce the relative ketone specific activity.

Identical measurements also were made using liver slices from animals killed at the mid-light point of the light cycle as shown in the lower half of Table I. While, as expected, the absolute incorporation rates were lower than those found in animals in the mid-dark phase, there was a similar relative contribution of C₃ units from the mitochondrial and cytosolic acetyl-CoA pools to cholesterol synthesis.

Using calculations described in detail previously (13) these experimentally determined values were used to determine the magnitude of the flux of C₃ units through the mitochondrial and cytosolic acetyl-CoA pools as shown in Columns E, F, and G of Table I. In Column E, the incorporation rates of [1-¹⁴C]octanoate into cholesterol were multiplied by 4 so that both the acetate and octanoate data could be normalized to equivalent C₃ units. Since [1-¹⁴C]octanoate is incorporated into cholesterol by the cytosolic biosynthetic pathway only after its intramitochondrial oxidation to acetyl-CoA, the true flux of C₃ units into cholesterol from the mitochondrial acetyl-CoA pool could be calculated by...
correcting the observed flux rates of labeled C\textsubscript{3} units from [1-\textsuperscript{14}C]\-octanoate into cholesterol for dilution of the intramitochondrial acetyl-CoA pool by endogenously produced C\textsubscript{3} units as evidenced by the relative specific activity of ketone bodies (Column F). Since labeled C\textsubscript{3} units entering cholesterol from [1-\textsuperscript{14}C]acetate presumably did so directly without prior equilibration with the intramitochondrial C\textsubscript{3} pool it was not possible to correct these flux rates using the specific activity of the generated ketones: hence, no corrected values have been entered for acetate in Column F. Finally, in order to correct for loss of 33% of the radioactivity as [\textsuperscript{14}CO\textsubscript{2}] during sterol biosynthesis from [1-\textsuperscript{14}C]acetate, all of the incorporation rates for octanoate (Column F) and for acetate (Lines 1 and 2, Column E) were multiplied by a factor of 1.5 to give the values shown in Column G.

The rates shown in Column G provide direct quantification of the flux of C\textsubscript{3} units into cholesterol through the mitochondrial and cytosolic acetyl-CoA pools. Several points concerning these rates warrant emphasis. First, in studies using octanoate alone the radiolabeled precursor must be metabolized within the mitochondrion where it results in the rapid generation of acetyl-CoA. Intramitochondrial dilution of the specific activity of C\textsubscript{3} units derived from [1-\textsuperscript{14}C]octanoate can be corrected for using the specific activity of the newly synthesized ketones. Further dilution of the precursor specific activity in the cytosolic compartment presumably does not take place to a significant extent in this particular circumstance; hence, the calculated flux rates of C\textsubscript{3} units into cholesterol determined under these experimental conditions represent the most direct assessment of absolute rates of cholesterol synthesis in these slices.

Second, in the presence of both acetate and octanoate, the C\textsubscript{3} units incorporated into cholesterol are derived from both the activation of acetate in the cytosol and the generation of acetyl-CoA from octanoate in the mitochondria. Nevertheless, if HMG-CoA reductase is operating at a maximal rate, then the sum of the C\textsubscript{3} fluxes from [1-\textsuperscript{14}C]acetate and [1-\textsuperscript{14}C]octanoate into cholesterol should equal that flux rate found with [1-\textsuperscript{14}C]octanoate alone. As also illustrated by the data in the two studies in Table I, this was found to be the case. In the first experiment, the flux of C\textsubscript{3} units from [1-\textsuperscript{14}C]acetate (4 mM) into cholesterol in the presence of unlabeled octanoate (1 mM) equaled 98.5 nmoles g\textsuperscript{-1} hour\textsuperscript{-1}; while, in the opposite experiment, the flux of C\textsubscript{3} units from [1-\textsuperscript{14}C]octanoate (1 mM) into sterols in the presence of unlabeled acetate (4 mM) was 237.6 nmoles g\textsuperscript{-1} hour\textsuperscript{-1}. The sum of these two flux rates, 336.1 nmoles g\textsuperscript{-1} hour\textsuperscript{-1}, essentially equals that found with octanoate alone (349.1 nmoles g\textsuperscript{-1} hour\textsuperscript{-1}).

Third, in the presence of [1-\textsuperscript{14}C]acetate alone as substrate, the sum of the flux rates of C\textsubscript{3} units from [1-\textsuperscript{14}C]acetate into cholesterol and from the mitochondrial pool (trace labeled with [1-\textsuperscript{14}C]octanoate) into cholesterol also should equal the rate observed with [1-\textsuperscript{14}C]octanoate alone. Clearly, this is not the case in either of the experiments. In the first study, for example, the flux of C\textsubscript{3} units into cholesterol from [1-\textsuperscript{14}C]acetate was 180 nmoles g\textsuperscript{-1} hour\textsuperscript{-1} and from the mitochondrial pool equaled 94.4 nmoles g\textsuperscript{-1} hour\textsuperscript{-1}. The sum of these two values, i.e. 274.4 nmoles g\textsuperscript{-1} hour\textsuperscript{-1}, is significantly less than the rate found with [1-\textsuperscript{14}C]octanoate. Similar findings were observed in the second set of data. Hence, these results not only show significant dilution of the specific activity of the cytosolic acetyl-CoA pool in slices incubated with [1-\textsuperscript{14}C]acetate alone, but, in addition, they also suggest that some step prior to HMG-CoA reductase, e.g. the activation of acetate in the cytosol, may be partially rate-limiting for cholesterogenesis when acetate alone is used as substrate.

An alternative approach that has been employed by several investigators to determine absolute rates of cholesterogenesis makes use of the fact that during reductive biosynthesis tritium from [\textsuperscript{3}H]water becomes incorporated into the final products (2, 14, 15). Since the specific activity of intracellular water presumably does not change in different experimental situations, use of this substrate eliminates the errors associated with variation in the degree of dilution of the specific activity of the cytosolic acetyl-CoA pool encountered when radiolabeled acetate is used. However, this method does not yield absolute rates of cholesterogenesis unless one also has knowledge of the ratio of \textsuperscript{3}H atoms incorporated into the sterol molecule per carbon atom (2, 14), and this ratio has not been determined in the many physiological circumstances in which the rate of cholesterogenesis is of interest. Furthermore, because of the high molarity of water, very large amounts of radioactivity are required when \textsuperscript{[3]H]water is utilized so that this method becomes impractical when large numbers of samples are under study. For these reasons, the use of \textsuperscript{[3]H]water seems to us less satisfactory than the use of [1-\textsuperscript{14}C]octanoate to assay rates of cholesterogenesis in whole cell preparations.

Finally, it is of interest to consider the results of this study in the context of the long standing question as to whether the initial reactions involved in the synthesis of ketone bodies and cholesterol, i.e. the conversion of acetyl-CoA into HMG-CoA, actually share the same pools of intermediates. That this is unlikely to be the case might be predicted on the basis of the following considerations. First, acetoacetate formation is primarily an intramitochondrial event (10) whereas the reduction of HMG-CoA to mevalonic acid takes place on the microsomes (7). Second, because the inner mitochondrial membrane is believed to be impermeable to acyl-CoA derivatives (17), this would be expected to present a barrier to the outward transport of a molecule such as HMG-CoA. Third, because of the exceedingly high activity of HMG-CoA lyase in liver and the almost exclusive intramitochondrial location of this enzyme (3, 7, 16), it is difficult to visualize how significant quantities of HMG-CoA synthesized within the mitochondrion could escape the action of the lyase and be used alternatively for cholesterol biosynthesis. For these reasons the suggestion has been made that the entire sequence of reactions leading from acetyl-CoA to cholesterol probably takes place in the extramitochondrial compartment of the hepatocyte (4, 7, 8). Such a concept is supported by the finding of significant quantities of \textsuperscript{3}H-ketothiolase and HMG-CoA synthase in the cytosolic space of liver cells (7, 8, 16, 18). Moreover, the results of the present investigation are entirely consistent with this viewpoint.

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\textsuperscript{2} As discussed previously (13) these calculations are based on the following assumptions: first, that the acetyl-CoA produced from the metabolism of endogenous substrate and that resulting from the oxidation of [1-\textsuperscript{14}C]octanoate enter a common pool prior to the formation of acetoacetate and second, that this pool of acetyl-CoA feeds both the enzymes of ketogenesis in the intramitochondrial compartment and those involved in cholesterogenesis in the extramitochondrial space.
Limitations of Acetate as a Substrate for Measuring Cholesterol Synthesis in Liver
John M. Dietschy and J. Denis McGarry


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