THE INCORPORATION OF THE CARBOXYL CARBON FROM ACETATE INTO CHOLESTEROL BY RAT LIVER HOMOGENATES*

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It is well known that the digitonin precipitate from the non-saponifiable fraction of tissue lipides contains substances other than cholesterol. The importance of this fact for isotopic studies of cholesterol synthesis has been emphasized by Schwenk and Werthessen (1). Working with C14-labeled material synthesized from acetate by organ perfusions, they have shown that the cholesterol is accompanied by substances of considerably higher specific activity. A single passage through the dibromide reduced the specific activity of various samples by 50 to 84 per cent.

Bucher and coworkers (2, 3) have recently reported the incorporation of C14-labeled acetate into a digitonin-precipitable material by cell-free preparations of liver. The present studies provide evidence that this newly formed radioactive substance is predominantly cholesterol, unaccompanied by significant quantities of impurities. Certain variables are described which affect the rate of labeled cholesterol formation by these cell-free homogenates, and some of the conditions necessary for maximal C14 incorporation are defined.

EXPERIMENTAL

Animals—Female rats of the Wistar strain, weighing approximately 200 gm., were used throughout. They were sacrificed by decapitation.

Preparation of Tissue—The livers were excised rapidly, chilled on cracked ice, and ground gently at 300 r.p.m. for 1 to 1½ minutes with 2½ volumes of medium in a homogenizer of the Potter-Elvehjem type which consisted of a smooth glass tube and stainless steel pestle, with a gap of approximately

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0.5 mm. between pestle and tube. Unbroken cells and tissue debris were eliminated by centrifugation at $500 \times g$ for 10 minutes. This entire preparative process was carried out at 0°.

**Incubation Mixture**—The homogenization medium consisted of potassium phosphate buffer, magnesium chloride, and nicotinamide. Diphosphopyridine nucleotide (DPN) and labeled acetate were added to aliquots of the homogenate supernatant fluid prior to incubation, which was carried out in a Dubnoff shaker under 100 per cent oxygen at 37° for 2 hours. All incubations were run in duplicate.

**Extraction of Cholesterol**—After incubation the mixture was rinsed into a Pyrex test-tube with water, and an equal volume of trichloroacetic acid was added. Following centrifugation, the supernatant fluid was discarded, and 6 ml. of 15 per cent alcoholic KOH and 0.8 mg. of carrier cholesterol in 95 per cent alcohol were added to the precipitate. The tubes were sealed, and subsequent hydrolysis, petroleum ether extraction, and precipitation of cholesterol were carried out as previously described (4). Samples were counted as the solid digitonide (4).

**Protein Content of Incubation Mixture**—The amount of homogenized liver actually present in the homogenate supernatant solution varied slightly from one experiment to another because the quantity of unbroken cell debris discarded was not always the same. As an index of the tissue present in the incubation mixture, the protein content of the homogenate supernatant fluid was determined on duplicate aliquots as follows: The proteins were precipitated with an equal volume of trichloroacetic acid, the nucleic acids and lipides were extracted from the precipitate after centrifugation (5), and the protein residues were quantitatively rinsed into tared aluminum foil dishes with acetone and dried to constant weight at 100°.

**Examination of Sterol Fraction Recovered As Digitonide for Identity and Purity**—4 ml. of a homogenate of rat liver were incubated under the above conditions with 1.74 mg. of $C^{14}$-carboxyl-labeled sodium acetate containing a total of $3.26 \times 10^7$ c.p.m. Hydrolysis with alcoholic KOH and extraction with petroleum ether were carried out as previously described (4). After evaporation of the petroleum ether, the residue was dissolved in 25 ml. of 1:1 acetone-absolute ethanol. 20 mg. of cholesterol (purified via the dibromide) were dissolved in this solution, and 15 ml. of 0.5 per cent digitonin in 50 per cent ethanol were added. The digitonide was split (6), and the ether-soluble fraction was dissolved in acetone. An additional 2 gm. of purified cholesterol were added as carrier. The following procedures

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1 Use of the stainless steel pestle was suggested to us by Dr. R. G. Langdon and Dr. K. Bloch.
were carried out to test the hypothesis that the radioactivity was present as cholesterol: (a) fractional crystallization, (b) passage through the acetate, (c) passage through the dibromide, and (d) a solubility curve. As a test of the efficacy of these procedures, three of them were applied to a mixture containing approximately 95 per cent non-radioactive cholesterol and 5 per cent C\textsuperscript{14}-labeled cholestanol, a substance commonly accepted as one of the most difficult to separate from cholesterol. The labeled cholestanol was prepared by catalytic hydrogenation at room temperature and atmospheric pressure from C\textsuperscript{14}-cholesterol obtained from liver slices incubated with labeled acetate. The cholesterol was acetylated prior to hydrogenation. The catalyst used was 10 per cent palladium on charcoal.

Acetylations were carried out in pyridine solution at 0\textdegree.

Brominations were performed by the method of Schoenheimer (7), except that the dibromide was not recrystallized, a modification recommended by Schwenk and Werthessen (1). Schoenheimer’s directions for recovery of the cholesterol, involving reduction with sodium iodide were followed (7).

In the experiments on cholesterol purity, heavier samples, about 8.8 mg. of digitonide, were prepared for counting, in order to increase the accuracy of the weighings. Counts were corrected for self-absorption to a standard thickness of 8.8 mg. spread over an area of 3.7 sq. cm.

Results

Reproducibility of Methods

Recovery of Cholesterol from Homogenates by Precipitation with Trichloroacetic Acid—In order to permit extraction of the cholesterol from the entire 2.5 ml. of homogenate and yet keep the volumes during hydrolysis and extraction at conveniently small values, the cholesterol was concentrated by precipitation of the proteins and lipides with trichloroacetic acid, as stated above. To determine the validity of this procedure, quadruplicate analyses were carried out on a radioactive homogenate, with and without prior precipitation with trichloroacetic acid, with the following results: on hydrolysates of unaltered homogenate, 46.9, 45.7, 47.9, 46.7 net c.p.m.; on hydrolysates of trichloroacetic acid precipitates, 41.2, 44.0, 41.9, 41.9 net c.p.m. These results indicate that about 90 per cent of the cholesterol is carried down with the precipitate.

Protein Content of Homogenates—The duplicate determinations of protein dry weight agreed with a standard deviation of ±0.9 per cent. The equivalent fresh weight of liver was found to be approximately 5 times the protein dry weight. In most experiments the incubation mixture contained approximately 200 mg. of fresh liver (40 mg. of protein, dry weight) in a total volume of 2.5 ml.
Factors Affecting Rate of Incorporation

Composition of Incubation Mixture—The results of omitting the various components of the incubation mixture singly or in combination are summarized in Table I. In the absence of added MgCl₂ or nicotinamide the activity was very low, and in the absence of both nicotinamide and DPN it was reduced to near zero. In the presence of nicotinamide, omission of supplementary DPN dropped the incorporation to approximately 25 to 50 per cent of the maximum obtained with the complete system.

The effects of varying the concentration of MgCl₂ are shown in Fig. 1, of nicotinamide in Fig. 2, and of DPN in Fig. 3.

The pH curve is shown in Fig. 4. It is recognized that the buffer strength employed in these experiments was inadequate to maintain the

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Acetate × 10⁻³ M</th>
<th>Complete system, microatoms incorporated*†</th>
<th>Component omitted</th>
<th>Altered system, microatoms incorporated†</th>
<th>Altered Control†</th>
</tr>
</thead>
<tbody>
<tr>
<td>63</td>
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<td>0.133</td>
<td>MgCl₂</td>
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<td>6</td>
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<td>0.8</td>
<td>0.0488</td>
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<td>0.0037</td>
<td>8</td>
</tr>
<tr>
<td>100</td>
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<td>0.394</td>
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<td>0.0154</td>
<td>4</td>
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<tr>
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<td>0.0610</td>
<td>DPN</td>
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<tr>
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<tr>
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<td>0.459</td>
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<td>0.0280</td>
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<tr>
<td>58</td>
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<td>0.0610</td>
<td>Nicotinamide + DPN</td>
<td>0.0005</td>
<td>0.8</td>
</tr>
<tr>
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<td>0.8</td>
<td>0.187</td>
<td>“</td>
<td>0.0011</td>
<td>0.6</td>
</tr>
<tr>
<td>86</td>
<td>5.8</td>
<td>0.114</td>
<td>“</td>
<td>0.0000</td>
<td>0.0</td>
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<tr>
<td>87</td>
<td>12.0</td>
<td>0.383</td>
<td>“</td>
<td>0.0000</td>
<td>0.0</td>
</tr>
<tr>
<td>106</td>
<td>12.0</td>
<td>0.459</td>
<td>“</td>
<td>0.0046</td>
<td>1</td>
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</tbody>
</table>

* The complete system, prepared and incubated as described in the text, contained the following in a volume of 2.5 ml: homogenate supernatant fluid; potassium phosphate buffer, pH 7.4, 0.08 M; magnesium chloride, 0.0048 M; nicotinamide, 0.03 M; DPN, 0.0008 M; and acetate-1-C¹⁴ in the molarities shown in the second column.
† The acetate incorporation is expressed as microgram atoms of acetate carboxyl carbon incorporated into cholesterol per gm. of fresh tissue per hour (2).
‡ The incorporation obtained with the complete system is regarded as 100 per cent. The effects of omitting components of the system singly, or in combination, are expressed in the last column as per cent of the activity in the complete system.
Fig. 1. Effect of Mg\(^{++}\) concentration on incorporation of acetate carboxyl carbon into cholesterol, expressed as in Table I. Conditions as for the complete system with 0.012 M acetate as stated in Table I, except that MgCl\(_2\) was omitted from the homogenizing medium and added afterwards.

Fig. 2. Effect of nicotinamide concentration on incorporation of acetate carboxyl carbon into cholesterol, expressed as in Table I. Conditions as for the complete system with 0.012 M acetate as stated in Table I, except that nicotinamide was omitted from the homogenizing medium and added afterwards.

Fig. 3. Effect of DPN concentration on incorporation of acetate carboxyl carbon into cholesterol, expressed as in Table I. Conditions as for the complete system with 0.012 M acetate as stated in Table I, except that the DPN supplement was varied as shown.

Fig. 4. Effect of varied pH upon the incorporation of acetate carboxyl carbon into cholesterol, expressed as in Table I. The initial and final pH is shown for each point. The average pH during the 20 minute incubation period is plotted. The homogenate was prepared in the usual way and preincubation pH adjustments were made by addition of small volumes of KOH or HCl. Other conditions as in the complete system with 0.012 M acetate, as stated in Table I.
pH of these concentrated homogenates at a constant level. However, an increase in phosphate concentration, or addition of another buffer (bicarbonate or imidazole), also failed to maintain the level during the 2 hour incubation period. Accordingly, to minimize the drop, the incubation period was shortened to 20 minutes. The optimal pH range appears to lie between 6.8 and 7.2. Because of the drop during incubation the maxi-

![Fig. 5](image1.png)

**Fig. 5.** Effect of tissue concentration on the incorporation of acetate carboxyl carbon into cholesterol, expressed as in Table I. A concentrated homogenate (1:1.5) was prepared. Different amounts of homogenate supernatant fluid were pipetted into each pair of incubation vessels, all being made up to a final volume of 2.5 ml. with the homogenizing medium. Other conditions were as in the complete system with 0.012 M acetate, as stated in Table I. The tissue concentration is expressed in terms of dry weight of protein.

![Fig. 6](image2.png)

**Fig. 6.** Amount of acetate carboxyl carbon incorporated into cholesterol, expressed as in Table I, at different time intervals. Conditions as in the complete system with 0.012 M acetate, as stated in Table I.

maximal incorporation in 2 hours was obtained when the pH of the buffer was initially 7.4 to 7.6.

The effect of varying the amount of tissue in the incubation mixture is shown in Fig. 5. Homogenates made in the manner described above often differ in activity from one preparation to another; the curve shown is representative, but not necessarily characteristic of all homogenates tested. In all instances higher activities were found at high tissue concentrations, and negligible activity in dilute preparations, but the concentration at which the curve became flat varied.

**Time of Incubation**—The amount of acetate incorporated after various
intervals of time is shown in Fig. 6. After a slight initial lag, the rate was nearly linear for the first 2 hours and did not deviate greatly even after 4 hours.

Oxidation of Acetate to CO₂—The effect of increasing the acetate concentration upon the amount of acetate oxidized to CO₂ and upon the amount of acetate incorporated into cholesterol is shown in Fig. 7. The incorporation curve reached a plateau at an acetate concentration of 8 to 12 × 10⁻³ M, whereas the amount oxidized continued to rise. The optimal fraction of added acetate incorporated into the cholesterol of the tissue in the vessels during the 2 hour incubation period, on the steep initial portion of the curve, was 2.7 per cent. At the left edge of the plateau, the value had dropped to 1.2 per cent. In terms of the available acetate present 20 to 25 per cent was oxidized to CO₂ in the presence of 8 × 10⁻³ M acetate, whereas only 12 per cent was oxidized when the acetate concentration reached 20 × 10⁻³ M. The QO₂ (N) was 70 μl.
Tests of Identity and Purity of Homogenate Cholesterol

In three of the following experiments, presentation of the results obtained with cholesterol from the homogenate is preceded by a description of a similar control experiment with an artificial mixture containing approximately 5 per cent radioactive cholestanol and 95 per cent non-radioactive cholesterol.

Fractional Crystallization—Table II shows the results when the artificial mixture was subjected to fractional crystallization from acetone. A nearly 6-fold difference in specific activity was obtained between the first and last crops. The possibility that the high activity of Crop 3 was due to a radioactive impurity in the cholestanol is minimized by the agreement observed when the ratio of cholesterol to cholestanol was investigated by infra-red analysis. The latter results are summarized in Table III.

Similarly, four crops of crystals were collected from an acetone solution containing 2 gm. of cholesterol and the digitonin-precipitable radioactive material from a homogenate. The specific activity of the various crops is shown in Table IV. A barely significant (6 per cent) increase in activity is apparent in Crop 3. Crop 4, constituting only 0.25 per cent of the whole, showed a specific activity 17 per cent higher than the average of Crops 1 and 2. The solution was evaporated practically to dryness to obtain this crop.

Acetylation—From Crop 2, 160 mg. of homogenate cholesterol were acetylated, and the acetate was recrystallized from acetone. 5 mg. were dissolved in 2.5 ml. of 1:1 acetone-ethanol, along with 1 mg. of cholesterol. Cholesterol digitonide was precipitated. The radioactivity of the precipitate was 7.4 net c.p.m., or 0.6 per cent of that present as acetylated material from the homogenate. This experiment shows that the radioactive compound synthesized by the homogenate behaves like cholesterol in the respect that it is no longer precipitable with digitonin after treatment with acetic anhydride. Furthermore, after treatment with this reagent it is not carried down to an appreciable extent along with a cholesterol digitonide precipitate.

Hydrolysis with alcoholic KOH was carried out on 138 mg. of the cholesterol acetate. The cholesterol was recrystallized from acetone and precipitated with digitonin for counting. Duplicate measurements gave 237 and 234 c.p.m. per mg., representing an insignificant (3 per cent) loss in specific activity as a result of passage through the acetate.

Dibromination—In agreement with Schoenheimer's observations (based on non-isotopic experiments), we found passage through the dibromide quite effective in removing cholestanol from cholesterol. One passage reduced the cholestanol content of our mixture to 1.16 per cent, as judged by the C^{14} measurements. Two passages reduced the percentage to 0.15.
TABLE II
Radioactivity of Successive Crops of Sterols Crystallized from Acetone Containing
Cholesterol Contaminated with Approximately 5 Per Cent Radioactive
Cholestanol

<table>
<thead>
<tr>
<th>Crop No.</th>
<th>Weight of crop (gm.)</th>
<th>C.p.m. per mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.034</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>0.854</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>0.011</td>
<td>150</td>
</tr>
</tbody>
</table>

TABLE III
Per Cent Composition of Mixtures of Sterols Obtained by Fractional Crystallization

<table>
<thead>
<tr>
<th>Crop No.</th>
<th>By measurement of radioactivity</th>
<th>By infra-red analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Cholesterol 95</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Cholestanol 4.8</td>
<td>4.7</td>
</tr>
<tr>
<td>3</td>
<td>Cholesterol 82</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Cholestanol 18</td>
<td>20</td>
</tr>
</tbody>
</table>

The infra-red analyses were made by comparison of the absorption at 1050 cm\(^{-1}\) and 1036 cm\(^{-1}\), at which cholesterol and cholestanol, respectively, show absorption maxima. In view of the errors involved in a quantitative infra-red analysis of this sort, it is considered that the excellence of the agreement is partially fortuitous. The authors are indebted to Dr. Jesse F. Scott for the performance and interpretation of the infra-red analyses.

TABLE IV
Radioactivity of Successive Crops of Homogenate Cholesterol Crystallized from Acetone

<table>
<thead>
<tr>
<th>Crop No.</th>
<th>Weight of Crop (gm.)</th>
<th>C.p.m. per mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.208</td>
<td>245, 246</td>
</tr>
<tr>
<td>2</td>
<td>1.245</td>
<td>246, 238</td>
</tr>
<tr>
<td>3</td>
<td>0.293</td>
<td>266, 251</td>
</tr>
<tr>
<td>4</td>
<td>0.005</td>
<td>294, 300, 286</td>
</tr>
</tbody>
</table>

TABLE V
Effect of Passage through Dibromide on Radioactivity of Homogenate Cholesterol

<table>
<thead>
<tr>
<th>Times through dibromide</th>
<th>C.p.m. per mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>246, 238</td>
</tr>
<tr>
<td>Once</td>
<td>242, 245</td>
</tr>
<tr>
<td>Twice</td>
<td>240, 241</td>
</tr>
</tbody>
</table>
In a parallel experiment, 914 mg. of homogenate cholesterol from Crop 2 were brominated and recovered from the dibromide. The product was recrystallized and the procedure repeated. As is seen in Table V, the specific activity of all samples counted, from the starting material and from that passed through the dibromide once and twice, fell within a total range of 3 per cent.

**Solubility**—A solubility curve on the artificial mixture, with 81 per cent ethanol as solvent, provided strong evidence of inhomogeneity in that the specific activity of the dissolved sterols in the vessels containing large amounts of undissolved solid was only 20 to 50 per cent that of the starting material.

![Fig. 8. Solubility curve of radioactive cholesterol synthesized by homogenates.](http://www.jbc.org/)

**DISCUSSION**

Taken together, these results appear to leave little reason to doubt that these homogenates do indeed incorporate C\(^{14}\) from acetate into cholesterol. Fractional crystallization indicates that other radioactive substances are also present, but in much smaller quantities than in cholesterol obtained in the perfusion experiments of Schwenk and Werthessen (1).
Bloch and his coworkers (8) have shown that the carbon from acetate incorporated into cholesterol by liver slices is widely dispersed throughout the molecule. In view of the lability of many synthetic systems in homogenates, the fact that the radioactivity of the cholesterol in the present experiments continues to rise for many hours raises the question of whether in our system a total synthesis may not be occurring. Pending completion of degradative studies on homogenate cholesterol, the possibility must be kept in mind that the $^{14}$C is entering the molecule by way of an exchange reaction, or that only a partial synthesis is taking place.

SUMMARY

1. We have investigated the effects of altering the composition of the incubation mixture upon the incorporation of carboxyl carbon from acetate-$^{14}$C into cholesterol, and have found that with approximately 200 mg. of tissue the maximal uptake was obtained in 2.5 ml. of the following: 0.08 M potassium phosphate buffer, pH 7.4, 0.03 M nicotinamide, 0.0048 M MgCl$_2$, 0.0008 M DPN, and 0.012 to 0.016 M acetate.

2. The radioactive cholesterol formed by these homogenates was recovered as the digitonide. Examination of one batch of such material from the standpoint of radiochemical identity and purity by fractional crystallization, passage through the dibromide, acetylation, and solubility all yielded results compatible with the hypothesis that the radioactive material was largely cholesterol.

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BIBLIOGRAPHY

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