Catabolism in Vitro of Cholesterol

II. FURTHER STUDIES ON THE OXIDATION OF CHOLESTEROL BY RAT LIVER MITOCHONDRIA*

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Earlier communications (1, 2) have described preparations derived from rat liver which are capable of oxidizing the terminal methyl groups of the cholesterol side chain to carbon dioxide. The requisite enzymes (cholesterol oxidase) are all present in the mitochondrial fraction of liver homogenates. Unknown factor(s) present in the soluble portion of the homogenate (supernatant factor), must be added for optimal activity (3).

This paper describes modifications of the enzyme preparation for the purpose of tracing some intermediate stages in the production of bile acids and carbon dioxide from cholesterol. By means of this approach, acetone has been detected as a product of cholesterol catabolism. We also report some studies on the oxidation of cholesterol biosynthesized from acetate-1-C¹⁴ thus containing C¹⁴ in carbon 25 and on further cofactor requirements for cholesterol oxidation. A preliminary report of some of these findings has already been made (4).

EXPERIMENTAL PROCEDURE

Cholesterol-20-C¹⁴ was synthesized from norcholest-5-ene-3β-ol-25-one (2, 5) and purified via the digitonide and by final passage through an alumina column. Cholesterol labeled at carbon 25 inter alia (cholesterol-25-C¹⁴) was prepared biosynthetically by incubating sodium acetate-1-C¹⁴ with rat liver slices (6) and purified similarly. Sodium pyruvate-2-C¹⁴ and sodium octanoate-1-C¹⁴ were obtained from the Radiochemical Centre, Amersham, England.

Preparation of Mitochondria and SF—Sucrose-washed liver mitochondria were isolated from 12- to 18-week-old male Wistar rats as described earlier (2). These washed mitochondrial preparations were then treated by one of the following procedures:

(a) Salt-extracted by resuspension in 0.15 M potassium chloride, followed by centrifugation at 8500 x g for 10 minutes. The supernatant fluid so obtained was discarded. The aggregated mitochondria were then treated by one of the following procedures:

(b) Extracted, or "lysed," with water, by suspension in distilled water at 2° for 30 minutes before addition to the incubation medium.

(c) Treated with a dilute solution of carbon tetrachloride (1.6 x 10⁻⁵ M) in 10% sucrose solution. After standing for 10 minutes at 2°, the mitochondria were separated by centrifugation (8500 x g for 10 minutes). The mitochondria were resuspended in the aqueous sucrose containing 1.6 x 10⁻⁵ M carbon tetrachloride for a further 10 minutes and reisolated by centrifugation. They were finally suspended in 10% sucrose and added to the incubation medium.

(d) Directly resuspended in 10% sucrose solution. This preparation is subsequently referred to as "intact" mitochondria.

The soluble cofactor (SF) was prepared with one major modification. Microsomes were removed from the mitochondria-free homogenate by further centrifugation at 100,000 x g for 25 minutes. The particle-free supernatant fluid so obtained was then boiled and the precipitated protein removed by low speed centrifugation. This clear fluid was used directly as the source of the cofactor(s), SF.

Conduct of Incubations—Incubations were normally carried out at 37° with shaking in the presence of 2000 units of penicillin G (potassium salt), 1 mg of streptomycin sulfate, 25 mg of ATP (disodium salt), 4 mg of DPN, 5 mg of AMP, 10 mg of magnesium nitrate (hexahydrate) and either 22 mg of trisodium citrate dihydrate (75 μmoles) or 75 μmoles of Tris-1-malate. Cholesterol emulsions in Tris-HCl buffer, pH 8.5, were prepared as described previously (2). The volume of the final incubation medium was 12 ml, containing mitochondria derived from 2 g (wet weight) of original liver tissue.

Incubations with sodium pyruvate and sodium octanoate were conducted at pH 8.5 in Tris-HCl buffer in the presence of SF, the cofactors, and citrate or malate as described previously.

When production of acetone was to be measured, salt-extracted mitochondria were incubated with the appropriate substrates in the absence of SF, GSH, and citrate (or malate). Zinc ions added as zinc sulfate at a final concentration of 10⁻⁵ M were usually added to increase the yield of acetone. The other cofactors (Mg²⁺, DPN, ATP, AMP) were added at half the above levels and the cholesterol was emulsified in 0.125 M Tris-HCl buffer.

After the incubation period (6 to 18 hours), the carbon dioxide
trapped in the alkali of the center wells was precipitated as BaCO₃ and counted in a liquid scintillation counting medium, as previously described (1).

Measurement and Degradation of Acetone and Acetoacetate—Acetone was isolated from the incubations by steam distillation without adjustment of the pH (at acid pH values, the steam volatility of cholesterol is increased). Water (40 ml) and sodium acetate (1.5 mg) were added to the incubation mixtures before distillation to serve as carriers. The distillates (approximately 10 ml) were refluxed for 30 minutes with mercuric sulfate in dilute sulfuric acid (Déniges reagent), cooled, and centrifuged. Acetone was recovered from the Déniges precipitates (mercury-acetone), by dissolving them in 2 ml of 2 N hydrochloric acid and water, followed by redistillation. Iodofom was prepared from these distillates with sodium hypochlorite. The mercury-acetone and iodofom precipitates were collected and their radioactivity measured in a gas flow counter.

Attempts to increase the yield of acetone and acetoacetate by oxidizing what might be considerable amounts of radioactivity in the form of β-hydroxybutyrate also resulted in some chemical degradation of the unmetabolized cholesterol to acetone. Incubations with boiled enzyme were always used as controls and similarly handled.

Acetoacetate, present in the incubation mixtures, was degraded as follows. Respiratory carbon dioxide was removed with a stream of alkali-washed air after acidification to pH 4.5 with 0.25 M succinic acid. Aniline succinate and carrier sodium acetoacetate (3 mg) were then added and the carbon dioxide, liberated by the aniline-catalyzed decarboxylation of acetoacetic acid, was trapped in sodium hydroxide and precipitated with barium chloride. The barium carbonate was counted in a liquid scintillation counter as described (2).

RESULTS

Radioactive carbon dioxide was formed on incubating cholesterol-25-C¹⁴ with suitable fortified rat liver mitochondria. Because of a lag phase, somewhat lengthy incubation periods (6 hours or more) are required for significant yields of carbon dioxide from cholesterol-25-C¹⁴ or 26-C¹⁴. There was no production of CO₂ from either of these substrates when boiled mitochondria were used, even when the incubation period was prolonged to 21 hours. The production of CO₂ can therefore be ascribed to the cholesterol oxidase activity of the liver mitochondria and not to bacterial contamination.

Cholesterol was added to all incubations as a Tween 20 emulsion in Tris buffer. It is of interest that cholesterol solutions, prepared by Avigan’s method (7) with fresh human serum, were only feebly oxidized.

The extent of oxidation of cholesterol to carbon dioxide was consistently reproduced in parallel incubations. Thus, in one experiment, six identical incubations shaken simultaneously for 14 hours gave yields of radioactive carbon dioxide within the range of 5.7 to 6.3% (calculated as BaCO₃/cholesterol-26-C¹⁴).

Requirement for and Distribution of SF—Optimal oxidation of both cholesterol-25-C¹⁴ and cholesterol-26-C¹⁴ was generally obtained only when boiled (deproteinized) solutions of the soluble particle-free fraction (SF) of a liver homogenate were added to incubations with the liver mitochondria. The stimulatory activity of these liver extracts (SF) was retained on storage at −15° for periods of up to 4 months, and was partially retained after concentration to a syrup at 40° under reduced pressure. Similar activity could be extracted with distilled water from acetone powders of rat, chicken, and mouse livers.

It should be emphasized that the actual enhancement in production of carbon dioxide from cholesterol, observed upon adding preparations of SF, varied widely from one mitochondrial preparation to another. In several instances, addition of this supernatant fraction has not stimulated the cholesterol oxidase system at all. Boiled extracts of liver mitochondria possessed no enhancing activity.

This unknown cofactor(s) does not appear to be confined to liver tissue. Boiled particle-free extracts of human placenta, bovine adrenal cortex, sow ovary, rat testicle, and rat spleen also exhibited some stimulatory activity. These tissue extracts had little or no stimulatory effect upon the oxidation of sodium pyruvate and sodium acetate by the same preparations of rat liver mitochondria and therefore appear to be concerned primarily with cholesterol oxidation. Boiled extracts of other rat tissues (skeletal muscle, thymus, brain, kidney, blood, small and large intestine) and of beef pancreas and adrenal medulla, bakers’ yeast and acetic acid were either ineffective or inhibited cholesterol oxidation by rat liver mitochondria.

Modified Preparations of Rat Liver Mitochondria—When sucrose-washed mitochondria were further treated by one of the following procedures: (a) extraction with isotonic KCl; (b) exposure to a small volume of distilled water; and (c) exposure to carbon tetrachloride dissolved in an aqueous sucrose solution, the cholesterol oxidase activity was retained. These procedures were employed to render more permeable, or disrupt entirely, those membranes which enclose the mitochondrial particle. Table I details the result of a time study on the relative activities of mitochondria subjected to procedures a, b, and c when compared with an equivalent amount of the original sucrose-washed mitochondria. In several such experiments no appreciable loss of activity was suffered after these manipulations.

Attempts to solubilize mitochondria by treatment with sodium deoxycholate destroyed the cholesterol oxidase activity. Extensive extraction of the mitochondria with distilled water also led to a great loss of activity, even when the extracted mitochondria were recombined with the extracts.

Inhibitory Action of Boiled Microsomes—We previously described (2) the preparation of SF, the soluble cofactor(s) enhancing cholesterol oxidation. Essentially, this earlier preparation was a boiled extract of the liver homogenate remaining after removal of the bulk of the mitochondria and all denser particles

<table>
<thead>
<tr>
<th>Mitochondrial preparation</th>
<th>Radioactivity % as CO₂</th>
<th>N mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.9 2.7 7.7 22.4</td>
<td>3.2</td>
</tr>
<tr>
<td>KCl-extracted</td>
<td>0.7 4.1 7.0 19.2</td>
<td>2.7</td>
</tr>
<tr>
<td>Water-lysed</td>
<td>0.9 2.9 8.9 22.4</td>
<td>2.9</td>
</tr>
<tr>
<td>CCl₄-extracted</td>
<td>0.7 2.6 10.2 19.6</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Table I
Cholesterol oxidations by various rat liver mitochondrial preparations at different time intervals
Equivalent amounts of sucrose-washed mitochondria were submitted to the procedures described in the experimental section. Percentage of radioactivity recovered was computed as BaC²O₄⁻O₃ cholesterol-26-C¹⁴ taken.
by centrifugation. We have since found that boiled extracts of the microsomal fraction may severely inhibit the oxidation of both cholesterol and sodium octanoate to CO₂ by liver mitochondria. Therefore, all preparations of SF are now made by boiling essentially particle-free extracts of liver, obtained after centrifugation of the homogenate at 100,000 × g for 25 minutes. Among the metal ions contained in boiled microsomal extracts, we have identified calcium ions which are potent inhibitors of mitochondrial oxidation (2). Boiled extracts of the nuclear fraction also depressed cholesterol oxidation.

The microsomal fraction also contains a high proportion of the total liver cholesterol (8). Thus, the yield of radioactive CO₂ from labeled cholesterol could be diminished by dilution with nonisotopic cholesterol. Formation of Acetone from Cholesterol—Distillation at pH 8.5 of flask contents obtained by incubating cholesterol-25-C¹⁴ yielded a radioactive steam-volatile product. From the distillates were prepared a radioactive p-toluenesulfonylhydrazone (9) and a radioactive 2,4-dinitrophenylhydrazone. These possessed melting points and mobilities identical with those of authentic acetone hydrazones after paper ionophoresis in acetate, phthalate, and borate buffers over the pH range 3.5 to 9.8, as well as paper chromatography in the solvent systems, toluene-acetic acid-water (4:4:1 volume for volume) and phenoxycetanol-heptane (10).

On reflux of the distillates with Déniges reagent, radioactive precipitates of mercury-acetone were isolated, from which steam-volatile radioactivity could be recovered by solution in hydrochloric acid (Table II).

The pH optimum for acetone formation from cholesterol appears to be between pH 8.5 and 9.0. Examination of the various subcellular liver fractions, singly and in combination, revealed that mitochondria alone possessed the requisite enzymes for acetone formation from cholesterol. In order to obtain significant yields of labeled acetone it was necessary to (a) use salt-extracted liver mitochondria (from previously fasted animals); (b) supply zinc ions to a final concentration of 10⁻⁵ M; and (c) eliminate addition of the SF preparation as well as GSH. CUPr ions (10⁻⁴ M) strongly inhibited acetone formation from cholesterol.

Degradation of the acetone by the haloform reaction indicated that the methyl groups were labeled only when the acetone was derived from cholesterol-26-C¹⁴. Acetone from incubations with cholesterol-25-C¹⁴ (or sodium octanoate-1-C¹⁴) was not labeled in the methyl groups (Table III). The sodium acetate formed in the haloform reaction was radioactive in every instance.

Inasmuch as cholesterol itself is steam-volatile, it is not surprising that trace amounts of radioactivity contaminated the mercury-acetone precipitate; the haloform reaction yielded no radioactive iodoform in this instance.

After removal of all of the respiratory carbon dioxide, the incubation products were treated with aniline succinate at pH 4.5, to decarboxylate any β-ketoacids present. The carboxyl carbon of acetooacetate was radioactive only when octanoate was employed as the substrate (Table III). The small yield of radioactive carbon dioxide liberated on aniline treatment from incubations with cholesterol-25-C¹⁴ and cholesterol-26-C¹⁴ may represent some fixation of respiratory carbon dioxide into oxalacetate. At pH 8.5, these salt-extracted mitochondrial preparations incorporated up to 9% of added bicarbonate-C¹⁴ into the carboxyl groups of these β-ketoacids (oxaloacetate, etc.).

Role of Citrate and of GSH—Calcium ions inhibit the formation of both carbon dioxide (2) and of acetone in vitro from the terminal isopropyl group of the cholesterol side chain. These ions must be effectively removed from the system in order to retain significant cholesterol oxidase activity. This can not be achieved consistently with Versene (ethylene diaminetetraacetate), probably because of its efficient binding of other (required) metal ions. The activating effect of citrate or certain of its metabolic precursors (pyruvate, other intermediates of the tricarboxylic acid cycle) could not be simply attributed to its ability to bind calcium alone, i.e. a static role. Other agents capable of forming complexes of calcium (oxalate, tartrate, mucate, d-glucurate, and phytate, as either the sodium or the potassium salts) activated cholesterol oxidase to varying degrees but were always less effective than equivalent amounts of sodium citrate. With the exception of phytate, none of these compounds form strong complexes with the common metal activators of enzyme systems (transition metals, etc.).

Alternative activating functions of citrate, or its metabolic

### TABLE II

Factors influencing yield of radioactive acetone

KCl-extracted liver mitochondria was incubated (without SF) for 14 hours with cholesterol-26-C¹⁴ (34,000 c.p.m.) or sodium octanoate (17,000 c.p.m.).

| Addition            | Source of acetone from cholesterol | Source of acetone from cholesterol-
|---------------------|-----------------------------------|------------------------------------|
|                     | From cholesterol                  | From cholesterol-
|                     | From acetate                      | From acetate-
|                     | c.p.m.                            | c.p.m.                             |
| None                | 150                               | 2090                               |
| Zn⁺⁺, 10⁻⁵ M        | 95                                | 2140                               |
| Zn⁺⁺, 10⁻⁴ M        | 340                               | 2140                               |
| Zn⁺⁺, 10⁻³ M        | 144                               | 2140                               |
| Zn⁺⁺, 10⁻² M        | 97                                | 2140                               |
| Ca⁺⁺⁺, 10⁻⁴ M       | 18                                | 2140                               |
| GSH                 | 75                                | 1480                               |
| Boiled enzyme       | 36                                | 34                                 |

* Added at conclusion of incubation.

### TABLE III

Distribution of C¹⁴ in acetone formed from octanoate-1-C¹⁴, cholesterol-25-C¹⁴, and cholesterol-26-C¹⁴

KCl-extracted mitochondria was incubated with substrates in the absence of SF, GSH, and citrate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Radioactivity of substrate (c.p.m.)</th>
<th>Radioactivity of mercury-acetone (c.p.m.)</th>
<th>Radioactivity of iodoform (c.p.m.)</th>
<th>CO₂ from carboxylic acid of acetone (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium octanoate</td>
<td>14,500</td>
<td>2,500 (36)</td>
<td>14</td>
<td>2,320</td>
</tr>
<tr>
<td>Sodium octanoate</td>
<td>14,500</td>
<td>2,500 (36)</td>
<td>14</td>
<td>2,320</td>
</tr>
<tr>
<td>Cholesterol-25-C¹⁴</td>
<td>37,566</td>
<td>712 (232)</td>
<td>136 (19)</td>
<td>28</td>
</tr>
<tr>
<td>Cholesterol-26-C¹⁴</td>
<td>34,100</td>
<td>428 (36)</td>
<td>138 (19)</td>
<td>28</td>
</tr>
<tr>
<td>Cholesterol-25-C¹⁴</td>
<td>6,400</td>
<td>202 (47)</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Cholesterol-26-C¹⁴</td>
<td>5,400</td>
<td>132 (14)</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

* Values in parentheses represent radioactivity from work up of parallel incubations with boiled mitochondria.
precursors, could be to "prime" the tricarboxylic acid cycle, to facilitate generation of ATP, or both, as well as to favor the reduction of pyridine nucleotides, i.e. a dynamic role. It was found that L-malate was occasionally a more efficient activator of carbon dioxide production from cholesterol than a molar equivalent of citrate (Table IV). These observations suggest that the activation of the system by citrate may be due to such a metabolic role rather than to formation of complexes of calcium ions.

The GSH requirement for cholesterol oxidation (2) is apparently specific. It could not be replaced by oxidized GSH, nor by other thiols tested (cysteine, cysteamine, ergothioneine, thiglycolic acid, BAL, thiamine acid, CoA) or their related disulfides. GSH also significantly enhanced the oxidation of sodium pyruvate and sodium octanoate to carbon dioxide at pH 8.5. In contrast, addition of GSH depressed acetone formation from both cholesterol and sodium octanoate (Table II). GSH, therefore, appears to have no specific effect upon cholesterol oxidation per se, but is rather an activator of mitochondrial oxidation in general. Zinc ions at a concentration of $10^{-4}$ M depressed this GSH effect.

### Table IV

**Activation of cholesterol oxidation by L-malate**

Incubation was with sucrose-washed rat liver mitochondria and SF for 15 hours at pH 8.5. Trisodium citrate, 75 pmoles; Tris-n-malate, 75 pmoles.

<table>
<thead>
<tr>
<th>Addition</th>
<th>CO$_{2}$ produced in</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>2,608</td>
<td>3,252</td>
<td>1,077</td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>2,720</td>
<td>3,446</td>
<td>1,352</td>
<td></td>
</tr>
<tr>
<td>Cholesterol-26-C$^{14}$ input</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(c.p.m.)</td>
<td>2,960</td>
<td>4,218</td>
<td>1,455</td>
<td></td>
</tr>
</tbody>
</table>

### Table V

**Effect of TPN and malate on formation of acetone and of carbon dioxide from cholesterol and sodium octanoate**

KCl-extracted liver mitochondria were incubated with SF for 5 hours at pH 8.5. Cholesterol-26-C$^{14}$ (85,000 c.p.m.) or sodium octanoate-1-C$^{14}$ (17,800 c.p.m.). TPN, 2 mg; Tris-L-malate, 75 pmoles.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Radioactivity</th>
<th>From cholesterol</th>
<th>From octanoate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mercury-acetone-C$^{14}$</td>
<td>c.p.m.</td>
<td>c.p.m.</td>
</tr>
<tr>
<td></td>
<td>BaC$^{14}$O$_{3}$</td>
<td>From octanoate</td>
<td>From cholesterol</td>
</tr>
<tr>
<td></td>
<td>c.p.m.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>805</td>
<td>4,250</td>
<td>2,880</td>
</tr>
<tr>
<td>Malate</td>
<td>1,390</td>
<td>4,380</td>
<td>2,730</td>
</tr>
<tr>
<td>TPN</td>
<td>920</td>
<td>4,470</td>
<td>3,940</td>
</tr>
<tr>
<td>TPN + malate</td>
<td>1,370</td>
<td>4,160</td>
<td>3,940</td>
</tr>
<tr>
<td>Boiled mitochondria</td>
<td>1,370</td>
<td>4,160</td>
<td>3,940</td>
</tr>
</tbody>
</table>

**Effect of TPN and Malate**—In the previous paper (2), we reported some attempts to demonstrate a requirement for TPN or TPNH. These experiments failed to show any direct effect of TPN or TPNH generators upon cholesterol oxidation by "intact" mitochondria in repeating these experiments with KCl-extracted mitochondria, from which much of the soluble content (nucleotides, etc.) of intact mitochondria had been removed, we found that the formation of CO$_{2}$ from cholesterol is indeed stimulated by the addition of TPN. Tables V and VI show that in identical incubations, TPN stimulated cholesterol oxidation to a greater extent than was observed in the oxidation of either sodium octanoate or sodium pyruvate.

The apparent inhibition of pyruvate oxidation which is observed with TPN in the presence of L-malate (Table VI; see also Table VIII), probably indicates the activity of the "malic enzyme" under our experimental conditions. Pyruvate formed by oxidative decarboxylation of the malate under the influence of TPN is oxidized by the malic enzyme to oxaloacetate, which is in turn converted to oxaloacetate by malic enzyme, with the formation of CO$_{2}$ and NADH. Oxaloacetate is then converted to pyruvate by oxaloacetate decarboxylase, with the formation of CO$_{2}$ and NADH. The NADH is then oxidized by the mitochondrial respiratory chain, with the formation of ATP.

### Table VI

**Effect of TPN on oxidation of cholesterol and sodium pyruvate**

KCl-extracted rat mitochondria were incubated with SF for 14 to 18 hours. Additions were: TPN, 2 mg; trisodium citrate, 75 pmoles; trisodium isocitrate, 75 pmoles; Tris-L-malate, 75 pmoles. Substrates were cholesterol-26-C$^{14}$ and sodium pyruvate-2-C$^{14}$. Percentage was computed as the increase in BaC$^{14}$O$_{3}$ upon addition of TPN.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>In presence of</th>
<th>Increase in formation of CO$_{2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol</td>
<td>Pyruvate</td>
</tr>
<tr>
<td>1</td>
<td>Citrate</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>Citrate</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>No addition</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>Citrate</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>14</td>
</tr>
</tbody>
</table>

* Inhibition actually observed.
of this TPN-coupled enzyme, could thereby dilute the radiactive substrate. Further evidence for the functioning of the malic enzyme in these salt-extracted mitochondria at pH 8.5, was obtained by direct spectroscopic observation of the TPN reduction of added l-malate.

In five further experiments, each with a different preparation of salt-extracted mitochondria, TPN, and malate (quantities as in Table V) together increased acetone formation from cholesterol by, respectively, 60, 70, 110, 230, and 240%. In each instance, acetone formation from sodium octanoate in parallel incubations was decreased by the TPN and malate.

This stimulation of cholesterol oxidation by TPN and malate, separately and in combination, together with the activity of the malic enzyme suggest that formation of TPNH in situ is probably responsible for the activation of cholesterol oxidase by TPN and by malate.

Other Activators of Cholesterol Oxidation—Acetone formation from cholesterol was enhanced by ferrie ions and by nicotinic acid (added as the sodium, potassium, or Tris salts (Table VII). Ferrous ions also stimulated cholesterol oxidation and acetone formation. In view of the known instability of ferrous ions at pH 8.5, these latter effects are probably due to ferrie ions formed by spontaneous oxidation. Ferric ions in contrast had little or no effect upon acetone production from sodium octanoate.

DISCUSSION

These studies have shown that C18 as well as C16 and C17 of cholesterol can be oxidized in vitro to carbon dioxide. The additional finding that labeled acetone can be derived from C16, C18, and C17 confirms the notion that cholesterol may be degraded by liver mitochondria with a loss of these three carbon atoms. The evidence furthermore suggests that the acetone is directly produced by cleavage of the C18-C20 bond rather than by way of acetoacetate and subsequent decarboxylation. To invoke the latter mechanism, one would have to postulate a fixation of CO2 on a terminal methyl group (for which there is no present evidence) followed by cleavage to yield acetocetate, or intermediate formation of acetyl-CoA with subsequent condensation to acetocetate. It should be pointed out, however, that whereas cholesterol-25-C14 yielded carbonyl-labeled acetone, the carboxyl group of the acetoacetate derived from the same incubations contained essentially no C14. It should be emphasized that these same mitochondrial preparations were capable of synthesizing acetocetate from octanoate.

It is of interest, also, that C18 of cholesterol is converted to the carboxylic carbon of acetone, whereas C16 and C17 exclusively label the methyl groups of acetone. Such evidence appears to exclude the formation of acetyl-CoA by B-oxidation of a bishomo-cholecholic acid (a C17-carboxylic acid). Such an acid has been previously postulated to be an intermediate in the biogenesis of cholic acids (11). Whether this cleavage mechanism represents the major or only pathway leading to the formation of bile acids is open to some question, particularly in view of the evidence that trihydroxyprostanoic acid is readily converted to cholic acid (11).

The stimulation by TPN is not surprising in view of its known role in the enzymatic hydroxylation of steroids and aromatic compounds (12, 13). The marked stimulation observed when TPN is supplemented with l-malate is particularly significant inasmuch as it has been ascertained that the "malic enzyme" is active in these mitochondrial preparations even at the alkaline pH employed. It is therefore quite clear that TPNH is generated under these conditions, and functions as a cofactor in the oxidative transformation of cholesterol to bile acids and carbon dioxide. Similar effects of malate and TPN have been described for steroid hydroxylation by mitochondria of the adrenal cortex (14).

Finally, the distribution of the soluble factor (SF) required for cholesterol catabolism suggests a resemblance to the cohydroxylase of Tomkins (15), inasmuch as it is found in those tissues which hydroxylate and oxidize steroids.

SUMMARY

Rat liver mitochondria are able to oxidize carbon atoms 2, 25, and 27 of the cholesterol side chain to carbon dioxide. Cholesterol oxidation is stimulated by reduced triphosphopyridine nucleotide, ferric ions, and a soluble cofactor(s) prepared from liver, testis, ovaries, adrenal cortex, placenta, and spleen.

Acetone has been identified among the products of cholesterol catabolism. The labeling pattern indicates that the methyl carbons of acetone are derived from the terminal methyls of cholesterol, whereas C18 of cholesterol is converted to the carboxyl group of acetone. The implications of this metabolic pathway are discussed.

Acknowledgments—We are indebted to Dr. V. Pileggi for the gift of purified sodium phytate, and to Dr. J. L. Rabinowitz for the cholesterol-25-C14. We are especially indebted to Mr. Leon Gogolefs and Mrs. Martha Cottrell for assistance with some of these experiments. The United States Educational Commission in the United Kingdom provided a Fulbright travel grant to one of us (M.W.W.) and made these studies possible.

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