THE CONVERSION OF CHOLESTEROL-4-C\textsuperscript{14} TO ACIDS AND OTHER PRODUCTS BY LIVER MITOCHONDRIA

BY DONALD S. FREDRICKSON

(From the Laboratory of Cellular Physiology and Metabolism, National Heart Institute, National Institutes of Health, Bethesda, Maryland)

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Since the finding by Bloch, Berg, and Rittenberg that cholesterol could be converted to cholic acid in the dog (1), several groups have established bile acids to be the primary end-product of cholesterol degradation by the liver in the intact rat (2, 3), rabbit (4), and human (5). Most of the steps involved in the conversion are still unknown, although the experiments of Zabin and Barker (6) and Staple and Gurin (7) indicate that cholic acid may be formed from cholesterol through the loss of only the 3 terminal carbons. Bergström, Pääbo, and Rumpf have presented evidence that ring substitution may precede degradation of the side chain (8). Experiments utilizing liver preparations in vitro which can oxidize cholesterol to CO\textsubscript{2} appear to offer a reasonable approach to further clarification of the degradation mechanism, and several such systems have been described.

The oxidation of the terminal carbon of the cholesterol side chain by tissue slices prepared from several organs, including liver, was reported by Meier, Siperstein, and Chaikoff (9). A cell-free preparation, containing mouse liver mitochondria and a soluble cofactor, which can oxidize over 10 per cent of the added cholesterol-26-C\textsuperscript{14} to C\textsuperscript{14}O\textsubscript{2} has been reported from this laboratory (10, 11). A particulate system from rat liver which oxidized cholesterol to CO\textsubscript{2} has also been reported by Lynn, Staple, and Gurin (12). They have reported finding as products 25-dehydrocholesterol and a steroid aldehyde and acid containing the radioactive terminal carbon (13).

In this paper we wish to report the analysis of the degradation products following the incubation of cholesterol-4-C\textsuperscript{14} in the mouse liver mitochondrial system. The results indicate that this liver cell fraction is capable of the production of several acids from cholesterol. Several other products, including cholesterol esters and a neutral steroid tentatively identified as 25- or 26-hydroxycholesterol, also accumulate during the reaction and may be involved in the conversion to acidic steroids.

Materials and Methods

Reference Compounds—25-Hydroxycholesterol (\(\Delta^4\)-cholestene-3\beta,25-diol) was prepared by hydrolysis of the acetate. Three recrystallizations of the product yielded fine needles with a melting point of 181–181.5°; \([\alpha]_D^{24}\)
-38.3° (2 per cent in CHCl₃) (published data; m.p. 181.5–182.5° (14)), [α]₁⁰⁵D -38.6° (15). 25-Dehydrocholesterol was prepared from 25-hydroxycholesteryl acetate (14), and 7-hydroxycholesterol (Δ⁵-cholesten-3β,7β-diol) from cholesterol after the method of Fieser et al. (16). The product contained no material absorbing in the ultraviolet above 220 nm, reacted strongly with the Lifschiitz reagent, and had a melting point of 156–159°. Chenodeoxycholic acid was isolated from goose bile (17). Cholesterol-4-C¹⁴ was incubated as a serum albumin-stabilized emulsion with washed mouse liver mitochondria, the heat-stable soluble fraction of whole liver homogenate, adenylic acid, nicotinamide, diphosphopyridine nucleotide, glutathione, tris(hydroxymethyl)aminomethane buffer, and sucrose as described previously (11). No significant radioactivity was obtained in the KOH wells suspended within the flasks. This is in agreement with previous reports which indicated lack of conversion of cholesterol-4-C¹⁴ to C¹⁴CO₂ in liver slices (9) and in the whole animal (18). Cholesterol-26-C¹⁴ was incubated separately in a single flask in most experiments, and radioactivity recovered as carbon dioxide provided a measure of the side chain degradation occurring with each mitochondrial preparation used.

Lipide Extractions—Immediately after incubation, the contents of the flasks were either lyophilized and extracted three times with acetone-ethanol (1:1), or the aqueous mixtures were repeatedly extracted with methylene chloride followed by diethyl ether. Extractions were made at room temperature and solvents were evaporated under nitrogen by distillation in vacuo. Extracts obtained after saponifications at elevated temperatures were concentrated on the steam bath under nitrogen. All extracts were stored in sealed containers at -15° during the time elapsed before analysis.

Identification of Acids—The presence of the carboxyl group in appropriate fractions of the total lipide extract was established by (1) the extract-

¹ Cholesterol-4-C¹⁴ was obtained from Radioactive Products, Detroit, Michigan. This material, which had a specific activity of approximately 2.5 μe. per mg., was repeatedly purified by chromatography on alumina and recrystallization during the course of the experiments.
ability of the radioactivity from an ether solution by three extractions with equal volumes of 0.4 M Na₂CO₃ and (2) precipitation with carrier cholic acid as the iron salt complex described by Doubilet (19). None of the acidic radioactive material was steam-volatile. The acids were further separated by paper chromatography or by counter-current distribution.

**Paper Chromatography**—Bile acids were separated on the collidine-water system of Siperstein, Harold, Chaikoff, and Dauben (20), or the systems of Sjövall (21) which employed (a) isopropyl ether-heptane (60:40)-HOAc (70 per cent), (b) isopropyl ether-heptane (20:80)-HOAc (70 per cent), (c) n-butanol-HOAc (70 per cent). Neutral steroids were separated on Quilon-treated paper (22), or by the system of Neher and Wettstein (23). One chromatographic system not previously described was developed to separate cholesterol and derivatives containing an additional free carbonyl or hydroxyl group. For this purpose, development on Quilon paper with solvents of greater polarity than the 80 per cent ethanol used by Kritcheksky and Calvin (22) was made possible by addition of benzene vapor from a wick placed in the chromatographic chamber. This provided the desired separations with excellent resolution not obtainable by the direct addition of benzene to the mobile phase. Cholesterol and 25-dehydrocholesterol were more completely separated by the Neher-Wettstein system. However, in the attempted identification of 25-hydroxycholesterol among the products, the short movement of this compound in the latter system, even after 30 hours development, rendered the Quilon system more valuable in determining the distribution of radioactivity. Radioactivity was localized by radioautographs or by counting the paper directly as small strips placed on steel planchets in the gas flow counter. The position of reference compounds was located after development by spraying the dried paper with phosphomolybdic acid or antimony pentachloride (24).

**Other Methods**—Except for those counts made directly on chromatographic strips, radioactivity was assayed by counting material as dried films on stainless steel planchets in a Robinson gas flow counter (25). The counts per minute were corrected to a sample absorption weight of 3.1 mg. per sq. cm. Ketonic steroids were isolated as derivatives of 2,4-dinitrophenylhydrazine or as mercuric iodide salts of the Girard hydrazone (26). Non-ketonic steroids were washed from the precipitate of the Girard salt by solution in acetone-ethanol and reprecipitation with heptane three times. Dehydrocholic acid (20 to 30 mg.) was added as carrier in each of the Girard Reagent T reactions.

**EXPERIMENTAL**

**Isolation and Separation of Products**—After preliminary experiments had indicated that significant amounts of cholesterol were being converted to both acidic and neutral products in the presence of intact mitochondria,
a relatively large scale experiment was performed in which 3.2 mg. of cholesterol-4-C\textsuperscript{14} (4.6 \times 10\textsuperscript{6} c.p.m.) were incubated with mouse liver mitochondria for 3 hours. The C\textsuperscript{14}O\textsubscript{2} trapped concurrently in the flask containing cholesterol-26-C\textsuperscript{14} contained 5 per cent of the radioactivity of the added substrate cholesterol. Following incubation, the contents of the flasks containing the ring-labeled cholesterol were pooled and essentially all (98.5 per cent) of the incubated radioactivity was recovered in the acetone-

![Flow diagram for separation of the radioactive products from the incubation mixture.](http://www.jbc.org/)
cent was removed from the acidified aqueous hydrolysate by heptane (Fraction II) and ether (Fraction III). The radioactivity remaining in the aqueous phase (Fraction IV) was reextracted with acetone-ethanol. Half of these counts proved to be digitonin-precipitable, and the remainder acidic. They were not further characterized. Thus, over 10 per cent of the cholesterol-4-C\textsuperscript{14} was converted to saponifiable material during the incubation.

**Acidic Products**—The radioactivity in the ether extract, Fraction III, was essentially all alkali-extractable, and precipitable as the Doubilet salt. It was then repeatedly separated into three bands, designated as acid

<table>
<thead>
<tr>
<th>Component</th>
<th>Fraction of substrate radioactivity</th>
<th>Digitonin-precipitable* per cent</th>
<th>Ketonic per cent</th>
<th>Polarity compared to known acids†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band A</td>
<td>0.1</td>
<td>80</td>
<td>100</td>
<td>&gt;Cholic</td>
</tr>
<tr>
<td>&quot; B</td>
<td>0.8</td>
<td>0</td>
<td>0</td>
<td>Cholic</td>
</tr>
<tr>
<td>&quot; C</td>
<td>1.9</td>
<td>35</td>
<td>20</td>
<td>Deoxycholic</td>
</tr>
<tr>
<td>Fraction II</td>
<td>6.6</td>
<td>0</td>
<td>60</td>
<td>Minimum of 3 components;‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>bulk = deoxycholic</td>
</tr>
</tbody>
</table>

* Digitonin precipitations were carried out in 80 per cent ethanol in the presence of carrier cholesterol. After 24 hours, the precipitates were washed three times with acetone-ethanol and the combined supernatant fluid and washes assayed for radioactivity.

† Comparison of polarity based on comparative \( R_F \) values obtained with paper chromatography or by counter-current separation.

‡ Of the Fraction II radioactivity, 2 per cent was retained at constant specific activity through six recrystallizations with authentic lithocholic acid.

Bands A, B, and C, by paper chromatography on the collidine-water system. When the bands were eluted and rerun, each maintained its characteristic \( R_F \). The \( R_F \) values of Bands B and C were identical to those obtained with reference to cholic and deoxycholic acids, respectively. Two bands, corresponding to Bands B and C, were likewise obtained when the Sjövall system I was used to separate Fraction III. However, recrystallization with authentic compounds and further chromatography of acid Band C on the Sjövall system II established that these acidic products were not identical to cholic, deoxycholic, or chenodeoxycholic acid. Further chemical behavior of acid Bands A, B, and C is summarized in Table I. None of the acids were available in sufficient quantities for infra-red analysis.

**Fraction II**—Because of the much less favorable solubility properties
of heptane for the common bile acids, the radioactivity in Fraction II was analyzed separately. Although a small fraction of the counts was found to represent cholesterol, over 90 per cent of the activity was alkali-extractable, and precipitable as the Doubilet salt. As indicated in Table I, none of the acidic radioactive material was digitonin-precipitable, but the presence of a mixture of acids was suggested by the result of the Girard Reagent T reaction, 60 per cent of the activity being precipitated as the hydrazone. Most of the radioactivity comprised a single broad component on the colloidine-water system corresponding to deoxycholic acid and acid Band C of Fraction III. Fraction II was further separated by a twenty-five tube counter-current distribution, the system n-heptane-HOAc (97.5 per cent) developed by Ahrens and Craig (27) being used. Three radioactive peaks were obtained with approximate K values of 0.01, 0.17, and 0.20. K for bile acids obtained by Ahrens and Craig with this solvent system ranged from 0.004 for cholic acid to 0.24 for lithocholic acid. None of the longer chain fatty acids had a K value of less than 1.9 (27). None of the Fraction II radioactivity was retained upon recrystallization with either cholic or deoxycholic acid, although a fraction of the counts remained with lithocholic acid through six recrystallizations (Table I).

Neutral Products of Degradation—Experiments preliminary to the large scale incubation just described also indicated the presence of at least two major neutral products. In these experiments, radioactivity was recovered from the incubation mixtures by extraction with methylene chloride and then ether. The combined extracts were dried, taken up in ether, washed with alkali and water, dried again, and taken up in benzene and chromatographed on alumina as shown in Table II. Highly significant radioactivity was recovered in the benzene and acetone eluates from material isolated after incubation of cholesterol-4-C\textsuperscript{14} with intact mitochondria, but essentially none after control incubations containing boiled mitochondria. The experiments in Table III confirmed the absence of non-enzymatically produced derivatives and suggested that the absence of the "soluble cofactor" (11) tended to decrease the production of the more polar derivative contained in the acetone eluate, while it enhanced the conversion to the less polar product. Partial identity of the neutral products was then established.

Cholesterol Esters—The benzene eluate was dried, taken up in heptane, and rechromatographed on alumina. Over 90 per cent of the activity was eluted with the less polar combination of benzene-heptane (1:9). When chromatographed on Quilon-treated paper and developed for 24 hours with 70 per cent ethanol in the presence of benzene vapor, all of this radioactivity remained discretely at the origin. None of the eluted material was precipitable with digitonin. After hydrolysis in methanolic KOH,
### Table II

**Alumina Chromatography of Neutral Steroid Products of Cholesterol Degradation**

<table>
<thead>
<tr>
<th>Eluate</th>
<th>Component steroids*</th>
<th>Eluate radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experimental</td>
</tr>
<tr>
<td>Benzene</td>
<td>Esters, cholestenone</td>
<td>6.9</td>
</tr>
<tr>
<td>Ethyl acetate-benzene (1:19)</td>
<td>Cholesterol (?)</td>
<td>7.5</td>
</tr>
<tr>
<td>&quot; &quot; (1:4)</td>
<td>Cholesterol, dihydrocholesterol, 25-dehydrocholesterol, 25-ketonorcholesterol</td>
<td>193.3</td>
</tr>
<tr>
<td>&quot; &quot; (1:4)</td>
<td></td>
<td>3.5</td>
</tr>
<tr>
<td>&quot; &quot; (1:4)</td>
<td></td>
<td>2.4</td>
</tr>
<tr>
<td>Acetone</td>
<td>25-Hydroxycholesterol</td>
<td>17.2</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td>0.8</td>
</tr>
</tbody>
</table>

* Determined by separate chromatography with authentic compounds.
† Control flasks contained boiled mitochondria.

Alkaline alumina (5 gm.) was employed in columns 13 cm. high and 0.9 cm. in diameter. The alumina was washed with acetone, then benzene, and charged with a benzene solution of the unknowns. All elutions were 50 ml. in volume.

### Table III

**Percentage Conversion of Cholesterol-4-C\textsuperscript{14} to Products Isolated by Alumina Chromatography**

The values are given in per cent of incubated cholesterol-4-C\textsuperscript{14} recovered.

<table>
<thead>
<tr>
<th>Alumina eluate</th>
<th>Flask 1</th>
<th>Flask 2</th>
<th>Flask 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact mitochondria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble cofactor, +</td>
<td>5.0</td>
<td>9.3</td>
<td>0†</td>
</tr>
<tr>
<td>Benzene</td>
<td>13.6</td>
<td>7.2</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Represents the unhydrolyzed contents of flasks extracted with methylene chloride and ether following a 3 hour incubation. Chromatography was carried out as in Table II.
† Mitochondria heated to 100° for 10 minutes before incubation.

the bulk of the radioactivity became digitonin-precipitable and, when rechromatographed on the same Quilon system, moved with an $R_f$ identical to that of cholesterol. On this same system, authentic cholesteryl acetate, propionate, and butyrate moved from the origin toward the front, while the oleate, palmitate, and stearate esters remained at the origin. It was
then concluded that the enzymatically produced radioactivity in the benzene eluates represented ester derivatives of cholesterol, and possibly other similar sterols, probably containing fatty acids of chain lengths greater than 4 carbons.

25- or 26-Hydroxycholesterol—The radioactivity in the acetone eluates obtained during the earlier experiments and from Fraction I (Table II) appeared to be due to a single compound, as indicated by the presence of a discrete spot upon repeated paper chromatography, corresponding to the $R_f$ of 25-hydroxycholesterol. On the Quilon system the activity ran well in advance of cholesterol and 7β-hydroxycholesterol. Both the 7α and 7β epimers of this compound have been determined to be among the principal products of the autoxidation of cholesterol in colloidal suspensions (28, 29).

While not entirely satisfactory for location of radioactivity because of the slow movement of 25-hydroxycholesterol, repeated chromatography of the same material on the Neher-Wettstein system confirmed the absence of significant radioactivity in the regions characteristic of both 7α- and 7β-hydroxycholesterol (30). No radioactivity was obtained in the mercuric iodide salt of the Girard hydrazone when aliquots of the acetone eluate were treated in the presence of carrier 25-ketonorcholesterol. The suggestion of a side chain-hydroxylated product offered by paper chromatography and the absence of a carbonyl group were augmented by the results of several color reactions. When sprayed on paper with SbCl₅ (20 per cent in chloroform), the 3,7-diol compound turned an immediate blue color, while the 3,25-diol and large amounts of the acetone eluate were both bright pink. The Lifschütz reaction, adapted by Bergström for the quantitative determination of 7-hydroxycholesterol (28), was negative in the presence of 25-hydroxycholesterol and the unknown material, but strongly positive in the presence of the 3,7 diol, when quantities of all three which produced colored spots of equal intensity with antimony pentachloride were used.

The radioactive material in the acetone eluate of the neutral Fraction I was then recrystallized with authentic 25-hydroxycholesterol with the results presented in Table IV. Constant specific activity corresponding to three-quarters of the initial activity of the eluate was obtained on the fourth through the sixth recrystallizations. The final crystals had the same melting point as the starting material. Repeated crystallizations were performed twice more with material obtained both before and after hydrolysis of the original lipide extract with the same results.

Dehydration of Polar Product—A separate aliquot of Fraction I was chromatographed on alumina with 12 mg. of carrier authentic 3,25-diol. All the added carrier was recovered in the acetone eluate. To this eluate 121 mg. more of the pure 3,25-diol were added, and the mixture containing
approximately 14,000 c.p.m. was acetylated in absolute pyridine and acetic anhydride. The resulting acetate was recovered and recrystallized with more (100 mg.) unlabeled 25-hydroxycholesteryl acetate. The specific activity of the recrystallized acetate was 63.0 c.p.m. per mg. (theoretical, 60 c.p.m. per mg.). The acetate was then converted to 25-dehydrocholesterol by phosphorus oxychloride dehydration, followed by hydrolysis (14). The product was recrystallized once from absolute methanol. The final specific activity of the white crystalline product was 66.2 c.p.m. per mg. The melting point and the infra red spectrum of the product were identical with that of authentic 25-dehydrocholesterol. The retention of all the radioactivity through the steps of this dehydration supplements the recrystal-

### Table IV

<table>
<thead>
<tr>
<th>Recrystallization No.</th>
<th>Solvent</th>
<th>Specific activity c.p.m. per mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methanol</td>
<td>13.0</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>11.1</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>9.3</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>9.1</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>9.5</td>
</tr>
<tr>
<td>6</td>
<td>Ethyl acetate</td>
<td></td>
</tr>
</tbody>
</table>

* A total of 1958 c.p.m. contained in the acetone eluate of products chromatographed on alkaline alumina was recrystallized with 150 mg. of 25-hydroxycholesterol (theoretical specific activity = 13.1 c.p.m. per mg.).

Esterification of Neutral Product—The neutral Fraction I (Fig. 1) was compared by alumina chromatography before and after hydrolysis. Of the total neutral radioactivity, 3.4 per cent was present in the acetone eluate before and 7.9 per cent after hydrolysis. Only 1 per cent of the activity appeared in the “ester” fraction after hydrolysis, and 22.7 per cent before. Thus a portion of the side chain-hydroxylated product is esterified, either before or after oxidation of the side chain. It is noteworthy that as much as one-fourth of the total sterol present was enzymat-
ically esterified during the mitochondrial attack on cholesterol during 3 hours of incubation.

25-Dehydrocholesterol—This compound has been suggested as an early intermediate in mitochondrial cholesterol oxidation by previous work in this laboratory (11) and by Lynn et al. (13). When the radioactivity from the alumina fraction (Table II) containing carrier 25-dehydrocholesterol was chromatographed on a phenoxyethanol-heptane column capable of separating authentic cholesterol from 25-dehydrocholesterol, only a fraction of 1 per cent of the radioactivity was present in the peak containing all the unlabeled carrier 25-dehydro compound. Since the original substrate had not been chromatographed on this column prior to incubation, the significance of the few counts obtained was questionable. The conclusion appears valid that 25-dehydrocholesterol does not accumulate in significant amounts as an intermediate in the oxidation of cholesterol by this mitochondrial system.

Other Ketones—Appropriate portions of the alumina separations (Table II) of the neutral fraction were treated with 2,4-dinitrophenylhydrazine in the presence of carrier cholestenone and 25-ketonoocholesterol. No significant activity was present in the hydrazone of cholestenone and little in the 25-keto preparation, indicating that these ketones do not accumulate as products in the present system.

DISCUSSION

The present results indicate that the mitochondrial fraction of liver homogenates is capable of the conversion of cholesterol to a complex mixture of acidic steroids containing at least four components. The mouse liver was employed in preference to that of the rat because of higher rates of oxidation of cholesterol to CO₂ obtained with mouse liver. Fewer data are available for comparison of the acids normally contained in mouse bile with the products obtained. However, the character of the latter, particularly the presence of ketonic and digitonin-precipitable acids, suggests an incomplete degradation in this system. Of the four most common acids in mammalian bile, only small amounts of lithocholic acid were possibly present in the products. The close similarity in chromatographic behavior, but non-identity, of major components of the acids to cholic and chenodeoxy or deoxycholic acid could indicate the presence of similar ring substitutions but differences in side chain length. In this regard, the result is important of studies in progress with side chain-labeled cholesterol in amounts sufficient to verify the presence or absence of the terminal carbon among the several acid products.

2 Unpublished results.
While the data presented offer substantial proof that the enzymatically produced more polar neutral product is 25-hydroxycholesterol, unequivocal identification and clarification of its rôle as an intermediate in the production of bile acids await further experiments, including the possible isolation of carrier-free amounts for analysis. The participation of a β-hydroxylated side chain derivative has been suggested by Haslewood (32) and by Lynn et al. (13). The logical position for secondary hydroxylation during conversion to bile acids would appear to be carbons 24 or 26, as suggested by these workers. Fieser has isolated 25-hydroxycholesterol as an autoxidation product in older samples of cholesterol (33).

The presence of a liver cholesterol esterase in the microsomes has been described (34). The esterase activity of the mitochondria may represent contamination with this enzyme, or a separate mitochondrial enzyme. The rôle of such an esterase in cholesterol degradation may be more than incidental, possibly affording protection of the 3-hydroxyl group while other portions of the molecule are being oxidized. Of interest in this regard is the observation that a greater proportion of the labeled hydroxycholesterol than that of the labeled cholesterol was esterified during the incubation. No major products suggestive of the C-21 steroids have thus far been isolated in keeping with the previous findings, indicating that bile acids are the principal end-products of liver oxidation of cholesterol.

**SUMMARY**

1. Cholesterol-4-C\(^{14}\) has been incubated with a cell-free preparation from mouse liver which has previously been shown to oxidize the terminal carbon of cholesterol to CO\(_2\). The products of the degradation of ring-labeled substrate have been separated and partially characterized.

2. Cholesterol is converted to at least four separate acids by the liver mitochondria. Two of these acids are similar to, but not identical with, cholic acid and deoxycholic or chenodeoxycholic acid. A ketonic acid and at least one digitonin-precipitable acid are also produced.

3. An enzymatically produced neutral derivative of cholesterol, accumulating during the reactions, has been tentatively identified as 25- or 26-hydroxycholesterol.

4. A large fraction of the cholesterol and the neutral product are enzymatically esterified during incubation.

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