
MODE OF OXYGEN INCORPORATION*

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Incubation of cholesterol with a bovine adrenocortical mitochondrial acetone-dried powder preparation yielded (22R)-22-hydroxycholesterol (I), (20R,22R)-20,22-dihydroxycholesterol (II), and pregnenolone (III) which were conclusively identified by combined gas chromatography-mass spectrometry. Incubations with [4-14C]cholesterol yielded I, II, and III with specific activities (determined from partial mass-spectral scans) not significantly different from those of the used substrate or the cholesterol reisolated after the incubation, demonstrating that the isolated compounds arose mostly, if not entirely, from the substrate cholesterol. Incubations in an 18O-enriched atmosphere yielded I, II, and III with 18O at position C-22, C-20 and C-22, and C-20, respectively, providing evidence that the hydroxyl groups of the side chain of I and II and the C-20 oxygen atom of III originated from molecular oxygen. The distribution of the oxygen atoms in II after incubation with 16O2 and 18O2 (devoid of 18O18O) proved that the hydroxyl groups of the side chain of II were introduced from two different molecules of oxygen, consistent with a sequential hydroxylation of cholesterol. No (20S)-20-hydroxycholesterol was found. Incubation of I in an 18O-enriched atmosphere afforded II and III with 18O at C-20.

In previous studies, the enzymatic transformation of cholesterol in bovine adrenal preparations to (22R)-22-hydroxycholesterol, (20R,22R)-20,22-dihydroxycholesterol, and pregnenolone has been investigated using radiochemical techniques (1, 2). Although the characterization of the hydroxylated cholesterol derivatives has been adequately established by reverse isotope dilution techniques, they have not been isolated from incubations in sufficient quantities for mass spectrometric analysis. The two hydroxylated cholesterol derivatives have been isolated in crystalline form and identified by classical means from 5 kg of whole bovine adrenal glands (3). No significant (20S)-20-hydroxycholesterol, however, has been isolated from adrenals (4) or formed from cholesterol (1, 2, 4, 5). That (22R)-22-hydroxycholesterol and (20R,22R)-20,22-dihydroxycholesterol can serve as intermediates in the conversion of cholesterol to pregnenolone has been established by kinetic measurements using labeled substrates (1, 2, 5). However, analysis of the kinetic data indicated that the glycol may also arise “directly” from cholesterol and not only via the (22R)-22-hydroxycholesterol (2, 5). This apparent one-step transformation could either indicate an introduction of an intact molecule of oxygen, possibly through the intermediacy of a hydroperoxide (6, 7), or represent relatively rapid sequential hydroxylations on an organized multienzyme complex (8), possibly involving transient free radicals or ionic species (9). Kinetic studies on substrate-enzyme interaction tended to favor the consecutive hydroxylation scheme (10, 11). The question of whether the oxygen atoms of the glycol are derived from the same oxygen molecule or from separate molecules was recently resolved by incubation of cholesterol in the presence of 16O2 and 18O2 (devoid of 18O18O) (12). Mass spectrometric analysis of the formed glycol clearly established the random nature of the oxygen incorporation into the side chain of cholesterol. The present article constitutes a detailed account of our mass spectrometric studies on the hydroxylation of cholesterol and of its conversion to pregnenolone with bovine adrenocortical preparations. It describes the conclusive identi-
fication by GC-MS of (22R)-22-hydroxycholesterol and (20R,
22R)-20,22-dihydroxycholesterol as enzymatic hydroxylation
products of cholesterol, as well as the mode of incorporation of
oxygen into the side chains of cholesterol and of (22R)-22-
hydroxycholesterol, and into pregnenolone.

EXPERIMENTAL PROCEDURE

Adrenal Preparations Two bovine adrenocortical mitochondrial
tissue was ground in ice-cold 0.02 M potassium phosphate buffer
(4°C) using a Potter-Elvehjem homogenizer. The homogenate
and the oxygen in a total volume of 6000 ml. Incubation C used 4 mg of unlabeled cholesterol
containing 2.2 and 2.5 mg/ml of protein, respectively. Preparation 1 was used in incubations A, C, D, E, F, and 2 in
incubations B, G, and H.

Preparation I was used in incubations A, C, D, E, F, and 2 in
for 20 to 21 min. The final protein
concentrations in incubations A to H were 4.2, 1.4, 4.2, 4.2, 2.1, 2.1, 1.4,
and 2.1 μM, respectively. The concentrations of glucose-6-phosphate dehydrogenase in incubations A to H were 4.2, 1.4, 4.2, 4.2, 2.1, 2.1, 1.4,
and 2.1 μM, respectively. The concentrations of glucose-6-phosphate dehydrogenase in incubations A to H were 4.2, 1.4, 4.2, 4.2, 2.1, 2.1, 1.4,
and 2.1 μg/ml, respectively. The reactions were terminated by adding approximately 2 liters of
2% aqueous acetic acid (which had been flushed with nitrogen for
approximately 20 min) followed by vigorous shaking.

Isolation of Products for GC-MS—To the ethyl acetate extracts of
the products were purified at an efficiency of 25.6% for 4H and 61.0% for 4C with corresponding backgrounds of 20
and 13 cpm and a carry-over of 4C into the 4H channel of 0.13, and no
significant carry-over of 4H into the 4C channel.

Derivatization and GC-MS—The samples were dissolved in 10 μl
of reagent in 0.3-ml tared vials equipped with Teflon-lined screw caps.

The products were purified by high-performance liquid chromatography as trimethylsilyl ethers by reacting with bis(trimethylsilyl)acetamide.

GC-MS with an LKB 9000 instrument was used both for identification
and determination of isotope ratios (13C and 2H). The use of GC-MS in the determination of specific activities of 4C-containing
compounds has been described (13, 14). Cholesterol was examined as the free sterol. (20R)-22-Hydroxycholesterol and (20R,22R)-20,22-
dihydroxycholesterol were examined as di(trimethylsilyl) and tri(tri-
methylsilylimidazole and heated at 100° for 20 min to obtain the tri(tetra-
nyl)ether. The GC-MS with an LKB 9000 instrument was used both for identification
and determination of isotope ratios (13C and 1H). The use of GC-MS in the determination of specific activities of 1H-containing
compounds has been described (13, 14). Cholesterol was examined as the free sterol. (20R)-22-Hydroxycholesterol and (20R,22R)-20,22-
dihydroxycholesterol were examined as di(trimethylsilyl) and tri(trimethylsilyl) ethers, respectively. Pregnenolone was examined both as the free compound and as its trimethylsilyl ether. The GC column used
was a silanized glass column (6 ft × 0.25 in) containing 1% OV-1 on Supelcoport (100 to 120 mesh). Column temperatures were 210° for
pregnenolone and its trimethylsilyl ether, 230° for cholesterol, and 250° for trimethylsilyl ethers of the hydroxylated derivatives. The flash heater and separator temperatures were 270° and 250°, respectively, and the electron energy was 22.5 eV. Some additional experiments were performed using a similar column containing 1% OV-17 main-
tained at 250° with all compounds. Complete mass spectral scans were obtained from 1 to 3 μg of material under normal conditions, i.e. scan speed, 6 or 7 (50 s to 20 s for total scan from 1 to 1000); filter, 120 cps; multiplier gain, 6; entrance slit, 0.03 mm; exit slit, 0.1 mm. Background ions were subtracted from line diagrams of the complete mass spectra. Isotope ratio measurements were performed on 50 to 500 μg of sample by making repetitive partial mass spectral scans over the mass range of interest as the compound emerged from the GC column. The higher sensitivity required for these determinations was attained by
operating the instrument under the following conditions: scan speed, 4;
filter, 60 cps; multiplier gain, 4 to 11 (as appropriate); entrance slit, 0.1 mm; exit slit, 0.5 mm. Mean isotope ratios were determined, unless otherwise stated, from at least 10 partial mass spectra obtained for each GC peak. In diagrams in which these data are illustrated, each
mark on the abscissa represents the first of 6 ions measured for each
can. No interfering background ions were encountered, and no
correction has been made for naturally occurring heavy isotopes, i.e. the
doubly or triply labeled exogenous compounds. Exogenous compounds were performed to obtain the empirical natural isotope ratios used to calculate 1H and 13C contents. The methods used to obtain the 13C and 1H contents of the various isolated products are more fully described and exemplified under “Results.”
RESULTS

Identification of (22R)-22-Hydroxycholesterol,
(20R,22R)-20,22-Dihydroxycholesterol, and Pregnenolone
Formed during Incubation with Cholesterol

Incubation G (total volume 6000 μl) was done with 4 mg of unlabeled cholesterol in air to obtain products which provided mass spectra without interference from species containing 14C or 16O for direct comparison with the authentic compounds. The relatively large incubation volume was necessary to obtain 2 to 4 μg of the hydroxylated cholesterol derivatives for a complete identification by GC-MS. Although the conditions used here were not obtained by strict optimization for the highest concentration of the hydroxylated cholesterol derivatives, the following data led to the choice of this experimental design. At the cholesterol concentration used (1.9 μM), maximal pregnenolone formation rates were realized, also giving approximately 4-fold higher concentrations of the hydroxylated cholesterol derivatives than at a cholesterol concentration of 0.14 μM. Although a 25-fold increase in the protein concentration led to an 18-fold increase in the concentration of pregnenolone, the concentration of the hydroxylated cholesterol derivatives did not increase significantly. The lower protein concentration was chosen because of the smaller possible interference by endogenous materials and because sufficient pregnenolone for GC-MS could be obtained at either protein concentration.

The mass spectrum of (22R)-22-hydroxycholesterol (obtained by GC-MS of the di(trimethylsilyl) ether) isolated from incubation G is given in Fig. 1 (lower) together with the spectrum of the authentic di(trimethylsilyl) derivative (upper). The unusual spectrum (15) is dominated by the ion of m/e 173, which comprises C-22 to C-27 (Scheme 1,b). This ion eliminates trimethylsilanol (90 a.m.u.) to yield the other abundant fragment ion at m/e 83. The molecular ion (m/e 546) was present only in low abundance (0.1%), and was accompanied by [M-1]+ (0.2%), [M-2]+ (0.1%), and [M-15]+ (0.3%). Minor differences in the mass spectra indicated that only traces of other compounds were present in the isolated material. It should be noted that none of the interfering ions is of relative abundance greater than 0.5% (the observed relative abundances for ions of m/e >200 in Fig. 1 have been increased 10-fold). The di(trimethylsilyl) ether had a similar retention index to that of the derivative of authentic material (Table I). In the partition systems used to isolate the (22R)-22-hydroxycholesterol, this compound separates from (22S)-22-hydroxycholesterol (1).

The mass spectrum of (20R,22R)-20,22-dihydroxycholesterol (obtained by GC-MS as the tri(trimethylsilyl) ether) isolated from incubation G is given in Fig. 2 (lower) together with the spectrum of the authentic tri(trimethylsilyl) derivative (upper). The base peak (m/e 461) comprises C-1 to C-21 (Scheme 1,a). This ion in turn affords fragment ions of m/e 371 and 281, respectively, by elimination of 1 and 2 molecules of trimethylsilanol (90 a.m.u.). The fragment ion of m/e 289 comprises C-20 to C-27 (Scheme 1,c). The molecular ion (m/e 634) is of very low relative abundance (0.05%) and is accompanied by a more abundant [M-15]+ ion (0.1%). There is no significant difference between spectra of authentic and isolated materials. The tri(trimethylsilyl) ether exhibited a retention index corresponding to that of the derivative of authentic material (Table I). In the partition systems used, (20R,-22R)-20,22-dihydroxycholesterol separates from the 22S analog.

The isolated pregnenolone from incubation G both as the free and its trimethylsilyl ether exhibited mass spectra (obtained from guest on October 15, 2017 http://www.jbc.org/ Downloaded from

We wish to thank Robert Suzuki for recording and interpreting the first spectra of the tri(trimethylsilyl) ether of authentic (20R,22R)-20,22-dihydroxycholesterol.

\[ \text{Scheme 1. Genesis of the ions of m/e 173, 289, and 461.} \]
tained by GC-MS) and retention indexes (Table I) corresponding to those of authentic materials.

No (20S)-20-hydroxycholesterol was detected by GC-MS in the combined fractions containing the tracer material added at the end of the incubation, after reaction with trimethylsilylimidazole. This finding was consistent with the absence of significant $^{14}$C in the purified (20S)-20-hydroxycholesterol fraction after incubation with [4-4$^{14}$C]cholesterol (see next section).

**Cholesterol as Precursor of (22R)-22 Hydroxycholesterol, (20R,22R)-20,22-Dihydroxycholesterol, and Pregnenolone Formed in Incubations**

Concurrent with incubation G was performed incubation B with [4-4$^{14}$C]cholesterol run at $\frac{1}{4}$ the scale but otherwise using the identical buffer, enzyme preparation, and cofactor solutions, as well as the identical isolation procedure. This experiment was done to ascertain that the compounds identified in incubation G were indeed formed from substrate cholesterol. To confirm the results of incubation B another similar incubation (A) was also done. The derivation of the $^{14}$C contents of the substrate and reisolated cholesterol and of the products are summarized in Table II. Also included in this table are the total amounts of product formed, obtained from their $^{14}$C radioactivity as corrected for procedural loss based on the recovery of the tritiated tracers added at the end of the incubations.

The $^{14}$C content of the substrate cholesterol was obtained from repetitive mass spectral scans in the molecular ion region during its elution from the GC column. The mass spectrum of the cholesterol reisolated from incubation B is shown in Fig. 3. Ions which contain C-4, notably M$^+$ (m/e 386), [M-15]$^+$ (m/e 371), [M-18]$^+$ (m/e 368), and [M-33]$^+$ (m/e 335) are accompanied by ions 2 a.m.u. higher. The $^{14}$C content of (22R)-22-hydroxycholesterol isolated from the incubations was obtained from mass spectral scans of the [M-15]$^+$ ion (m/e 331) of the di(trimethylsilyl) ether. Because only about 300 ng of this compound were available, only weak mass spectral data were obtained. In incubation B, the signal to noise ratio was approximately 5. Eight multiplets with relative abundances of ions of m/e 533 (compared to m/e 531) in the range of 0.63 to 0.82 were obtained. There was a contaminant which afforded an ion of m/e 535 with relative abundance similar to that of the ion of m/e 531. This contaminant was not present in incubation A. The data summarized for incubation A in Table II represent the mean of three multiplets with relative abundances of m/e 533 (compared to m/e 531) in the range of 0.71 to 0.75. The $^{14}$C content of (20R,22R)-20,22-dihydroxycholesterol was obtained from repetitive mass spectral scans of the C$_1$ fragment ions of the m/e 461 region, given in Fig. 4. The first, and most abundant, peak in each group is m/e 461 and the next most abundant is m/e 463. The molecular ion (m/e 316) was used to determine the $^{14}$C content of the isolated pregnenolone. No significant $^{14}$C was present in the (20S)-20-hydroxycholesterol fractions from incubations with [4-4$^{14}$C]cholesterol.

Incubation E was accompanied by a blank incubation with [4-4$^{14}$C]cholesterol and $^{18}$O, but with no enzyme, done under the same conditions as the other incubations concerning duration and TPNH concentrations. No pregnenolone or hydroxylated cholesterol derivatives were found by $^{14}$C counting or repetitive mass spectral scanning, in which only background ions were seen.

The $^{14}$C content of the compounds as obtained by correcting for heavy isotope contribution is summarized in Table II. The correction factors for heavy isotope contribution which were used in the calculations were 0.06 for cholesterol (m/e 386), 0.17 for (22R)-22-hydroxycholesterol di(trimethylsilyl) ether (m/e 531), 0.15 for (20R,22R)-20,22-dihydroxycholesterol tri(trimethylsilyl) ether (m/e 461), and 0.03 for pregnenolone (m/e 316). Calculation of $^{14}$C content is exemplified for substrate cholesterol (Table II). The corrected abundance of the ion of m/e 388 is $0.402 - 0.06 \times 0.598 = 0.366$, and the corrected

![Fig. 2. Mass spectra of the tri(trimethylsilyl) derivatives of authentic (upper) and isolated (lower) (20R,22R)-20,22-dihydroxycholesterol produced by incubation of cholesterol in air (incubation G).](http://www.jbc.org)
Table II

Partial mass spectral data for \(^{4-14}\text{C}\)cholesterol and its products and calculation of \(^{14}\text{C}\) content

The total amounts of the products formed are also given. The column labeled “measured abundance” represents the mean values of actual measured peak heights, and the data in the column labeled “corrected abundance” have been corrected for naturally occurring heavy isotopes. Specific activities have been calculated from the \(^{14}\text{C}\) content (corrected abundance of the ions 388, 533, 463, and 318) and the specific activity of \(^{14}\text{C} = 82.4 \text{Ci/g atom.}\)

![Normalized abundances of ions of m/e 386 and 388 are 0.620 and 0.380 giving a \(^{14}\text{C}\) content of 38.0%.](https://example.com)

It may be seen from Table II that the specific activity of the cholesterol reisolated from the incubation and those of the products were not significantly different from that of the substrate cholesterol in both incubations A and B.

Origin of Oxygen Atoms Incorporated into Hydroxylated Cholesterol Derivatives and Pregnenolone

Incorporation of \(^{18}\text{O}\) into Cholesterol Side Chain and Pregnenolone

These studies were done using \(^{4-14}\text{C}\)cholesterol as substrate. From the \(^{14}\text{C}\) content (as determined by liquid scintillation spectrometry) the amount of product available was known, allowing the proper setting of the instrument for obtaining the highest intensity mass spectral scans. The composition of the atmospheres used in Experiments C to F and the summary of the calculation of the \(^{18}\text{O}\) contents of the isolated compounds are presented in Table III. No significant change in the isotopic composition of the oxygen in the atmospheres was observed during the incubations. In particular, there was no appearance of \(^{18}\text{O}^{16}\text{O}\) in the atmospheres during incubations using \(^{18}\text{O}_{2}\) and \(^{18}\text{O}_{2}\). Included in Table III are also the total amounts of products formed obtained from their \(^{14}\text{C}\) radioactivity, corrected for procedural loss based on the recovery of tritiated tracers added at the end of the incubations.

<table>
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<tr>
<th>Compound</th>
<th>Incubation</th>
<th>Ion m/e</th>
<th>Measured Abundance</th>
<th>Corrected Abundance</th>
<th>Specific Activity (Ci/mole)</th>
<th>Total Product (μg)</th>
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<tbody>
<tr>
<td>Cholesterol substrate</td>
<td>A,B</td>
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<td>0.598</td>
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<td></td>
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<td>388</td>
<td>0.402</td>
<td>0.380</td>
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<tr>
<td>Cholesterol (reisolated from the incubation)</td>
<td>A</td>
<td>386</td>
<td>0.602</td>
<td>0.624</td>
<td>23.5</td>
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<tr>
<td></td>
<td></td>
<td>388</td>
<td>0.398</td>
<td>0.376</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>386</td>
<td>0.602</td>
<td>0.624</td>
<td>23.5</td>
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<tr>
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<td>388</td>
<td>0.398</td>
<td>0.376</td>
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<td>(22R)-22-Hydroxycholesterol d1-TMS</td>
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<td></td>
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<td>0.648</td>
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<td></td>
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<td>533</td>
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<td>(20R,22R)-20,22-Dihydroxycholesterol tri-TMS</td>
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<td>0.627</td>
<td>23.3</td>
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<td></td>
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<td>463</td>
<td>0.427</td>
<td>0.373</td>
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<tr>
<td></td>
<td>B</td>
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<td>463</td>
<td>0.437</td>
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<td>Pregnenolone</td>
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<tr>
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<td>318</td>
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<td>0.378</td>
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1 Obtained from the \(^{14}\text{C}\) radioactivity

![Mass spectrum of cholesterol reisolated after incubation of \(^{4-14}\text{C}\)cholesterol (incubation B).](https://example.com)
Cholesterol—As might be expected, there was no exchange of the oxygen at C-3 of cholesterol or of any of the other compounds studied.

(22R)-22-Hydroxycholesterol—The fragment ion of m/e 173 of the ditrimethylsilyl) derivative of this compound (Scheme 1) was used to determine the 18O content at C-22. In repetitive mass spectral scans of this compound isolated from an incubation of [4-13C]cholesterol in the presence of 18O2 (incubation A), it was found that the major ion in each multiplet was m/e 173 and the relative abundance of the ion of m/e 175 indicated, as would be expected, that it was due to naturally occurring heavy isotopes of hydrogen, carbon, oxygen, and silicon, with no contribution from fragment ions containing 13C. Partial mass spectral data for the ditrimethylsilyl ether of (22R)-22-hydroxycholesterol isolated after incubation with [4-13C]cholesterol in the presence of 18O2 (incubation A) are shown in Fig. 5. In this case, the ion of m/e 175 reflects the 18O content at C-22. The 18O content was obtained in a manner similar to that described in the text for the correction applied in Table II, using the empirical correction factor of 0.03 for naturally occurring heavy isotopes.

(20R,22R)-20,22-Dihydroxycholesterol—This compound contains 2 oxygen atoms in the side chain, each of which could derive from molecular oxygen. The ion of m/e 461 (Scheme 1a) of the tritrimethylsilyl ether contains the oxygen atom at C-20 as well as C-4. There are 3 major ions in each multiplet: ions of m/e 461 which derive from the [4-13C, 20-18O] compound; those of m/e 463 from the [4-13C, 20-18O] and [4-13C, 20-18O] analogs; and those of m/e 465 from the [4-13C, 20-18O] analog. In these experiments, a correction for 13C was required in addition to that for naturally occurring heavy isotopes to obtain the 18O content. Thus, the ion of m/e 463 is first corrected for naturally occurring heavy isotopes by subtraction of 0.15 of the abundance of the ion of m/e 461. The ion of m/e 465 is, in turn, corrected by subtraction of 0.15 of the corrected abundance of the ion of m/e 463. Contributions from 13C-containing species (0.380) were then subtracted. The calculation method is exemplified for incubation C (Table III). The normalized abundances (corrected for heavy isotopes) of ions of m/e 461, 463, and 465 are 0.321, 0.491, and 0.188. Of the molecules containing a 4-13C atom, 0.188 contain both 4-13C and 20-18O. The fraction of the species of m/e 463 containing 4-13C and 20-18O is, therefore, 0.380 - 0.188 = 0.192. The fraction of the molecules giving ions of m/e 463 containing the species [4-13C, 20-18O] is 0.491 - 0.192 = 0.299, and the fraction of the molecules containing a 20-18O atom is 0.188 + 0.299 = 0.487.

The fragment ion of m/e 289 of the glycol tritrimethylsilyl) ether was used to determine the mean 18O content in the side chain (Scheme 1c). Incubations with [4-13C]cholesterol and 18O2 (incubation A) demonstrated that there was no contribution of ions containing 18O to the ion of m/e 291. Repetitive scans in this region for incubation D (done in an atmosphere of 18O2 and 18O) are shown in Fig. 6. The first ion in each multiplet, at m/e 289, contains 18O2; the major ion, at m/e 291, contains 18O18O; whereas the ion of m/e 293 contains 18O. The empirical correction factor for naturally occurring heavy isotopes accompanying m/e 289 is 0.1. The calculation of the mean 18O content at C-20 and C-22 is exemplified for incubation C (cf., Table III). Ions of m/e 289, 291, and 293 have normalized corrected abundances of 0.269, 0.478, and 0.253. Because ions of m/e 291 contain 1 atom and those of m/e 293 2 atoms of 18O, the mean 18O content of the side chain is 0.478/2 + 0.253 = 0.492.

Pregnenolone—The 18O content of this compound was determined from its molecular ion region (m/e 316). The calculations of the 18O content were performed in a similar manner to those done for the glycol.

Because in some experiments, the 18O incorporation into pregnenolone was lower than that found in the hydroxylated cholesterol derivatives, the lability of the 20-keto group was investigated. [20-18O]Pregnenolone (12.5 μg from incubation H) was incubated for 40 min in air at 24°C with adrenal preparation 2 at a protein concentration of 0.2 mg/ml in a total volume of 6.0 ml at the usual TPNH concentration. At the end of the incubation, the pregnenolone was extracted with 20 ml of ethyl acetate and isolated by chromatography in system P-1. The recovered pregnenolone exhibited no dilution of the 18O label even though a higher protein concentration and a longer incubation time were used. This experiment suggested that loss of label may occur during the isolation rather than the incubation, especially when large volumes of ethyl acetate are used and special care is not taken to eliminate traces of acetic acid. When 10 μg of [20-18O]pregnenolone in 10 ml of water was acidified to pH 3 with acetic acid and left at 24°C for 90 min, there was a complete loss of the 18O label.

Incorporation of 18O into (20R,22R)-20,22-Dihydroxycholesterol and Pregnenolone Formed by Incubation of (22R)-22-Hydroxycholesterol

In this incubation (H) 12 μg of (20R,22R)-20,22-dihydroxycholesterol and 272 μg of pregnenolone were isolated, as calculated from the 3H radioactivity.

The mass spectrum (obtained by GC-MS) of the tritrimethylsilyl) ether of the isolated (22R,22R)-20,22-dihydroxycholesterol is shown in Fig. 7. The ion of m/e 463 clearly contains 18O, and the absence of an ion of m/e 293 shows that there is only one 18O atom in each molecule. Several additional interesting features may be seen. The ion of m/e 371 is not accompanied by an ion of m/e 373, and is most likely formed from the ions of m/e 463 and m/e 461 by elimination of trimethylsilanol from C-23. The ion of m/e 281 (which also lacks 18O) is most probably formed by a second elimination of trimethylsilanol from C-20. The ion of m/e 173 and the relative abundance of the ion of m/e 175 indicated, as would be expected, that it was due to naturally occurring heavy isotopes of hydrogen, carbon, oxygen, and silicon, with no contribution from fragment ions containing 13C.
The isolated pregnenolone from incubation H is shown in Fig. 8. It clearly contains $^{18}$O (m/e 318). The presence of the ion of m/e 45 confirms that the $^{18}$O atom is at C-20; the ion of m/e 43 has the structure CH$_3$C=O$^+$ containing C-20 and C-21.

The $^{18}$O content of the products was obtained from the full mass spectra which were adequate for this purpose because of the relatively high signal to noise ratio. The results are shown in Table IV.

**Summary of $^{18}$O Contents at C-20 and C-22 of Products**

The $^{18}$O contents expressed as fractions of the $^{18}$O composition of the incubation atmosphere are summarized in Table V.
The oxygen content at C-22 of the glycol was calculated from the ions in the region of m/e 289 and 461 as follows. The oxygen content of the side chain (A) determined from the ions of m/e 289, 291, and 293 is the mean of the oxygen atoms at C-20 (B) and C-22 (C): i.e. A = (B + C)/2. B is determined from the ions of m/e 461 and 463. The oxygen content of the oxygen atom at C-22 may then be calculated from the expression: C = 2A - B.

It may be seen from Table V that the oxygen incorporation from the atmosphere into the products was as high as 90%. The greatest variability was observed with pregnenolone as would be expected from the potential lability of the carbonyl group to exchange with water. The oxygen contents at C-20 and C-22 of the glycol were not significantly different in the incubations with cholesterol. In the incubation with (22R)-22-hydroxycholesterol only the oxygen atom at C-20 contained significant oxygen.

**DISCUSSION**

(22R)-22-Hydroxycholesterol and (20R,22R)-20,22-dihydroxycholesterol have been conclusively identified by mass spectrometry as products of the enzymatic hydroxylation of cholesterol in bovine adrenocortical mitochondrial preparations. The mass spectra of trimethylsilyl ethers of the isolated products were essentially indistinguishable from those of the authentic compounds and exhibited the presence of only minor impurities; there was excellent correspondence in the retention indexes by GC and, by virtue of the partition chromatographic systems used in their isolation, they were clearly distinguished from the corresponding 22S stereoisomers.

In the experiments with [4-14C]cholesterol as substrate, the specific activities (determined by mass spectrometry) of the hydroxylated derivatives and of pregnenolone were not significantly different from those of the substrate cholesterol or of the cholesterol reisolated from the incubations. The precision of the determinations was 2 to 5% (coefficient of variation as calculated from the respective mass spectral scans) for cholesterol.

![Figure 5](http://www.jbc.org/)

**FIG. 5.** Partial mass spectral data (m/e 173 to 178) obtained from repetitive mass spectral scans during elution from GC of the di(trimethylsilyl) derivative of (22R)-22-hydroxycholesterol isolated after incubation of [4-14C]cholesterol in the presence of 18O2 and 18O4 (incubation D).

![Figure 6](http://www.jbc.org/)

**FIG. 6.** Partial mass spectral data (m/e 289 to 294) obtained from repetitive mass spectral scans during elution from GC of the di(trimethylsilyl) derivative of (22R)-22-hydroxycholesterol isolated after incubation of [4-14C]cholesterol in the presence of 18O2 and 18O4 (devoid of 18O18O) (incubation D).

![Figure 7](http://www.jbc.org/)

**FIG. 7.** Mass spectrum of the tri(trimethylsilyl) derivative of (20R,22R)-20,22-dihydroxycholesterol isolated after incubation of (22R)-22-hydroxycholesterol in the presence of 18O2 and 18O4 (incubation H).

![Figure 8](http://www.jbc.org/)

**FIG. 8.** Mass spectrum of pregnenolone isolated after incubation of (22R)-22-hydroxycholesterol in the presence of 18O2 and 18O4 (incubation H).
terol, the glycol, and pregnenolone. The precision was lower (10%, coefficient of variation) for the (22R)-22-hydroxysterol because of the relatively low intensity of the ion that was used for determining its $^{14}$C/$^{12}$C ratio. Nevertheless, the mean observed for the reisolated cholesterol, and it is fair to conclude that all isolated products arose mostly (if not entirely) from the techniques in which the formation of a compound is established by hydroxylated cholesterol derivatives, incubations with total hydroxycholesterol and (20R,22R)-20,22-dihydroxycholesterol substrate cholesterol.

purification to radiochemical homogeneity by reverse isotope dilution techniques involving a series of chromatographic purification to constant specific activity (1). In order to obtain sufficient quantities for partial mass spectral scans of the radiochemistry to in vitro. The relatively small steady state concentrations of the hydroxylated cholesterol derivatives appear to be the result of their relatively high disappearance rates (10). No (20S)-20-hydroxycholesterol was observed in agreement with previous studies (1, 12, 4, 5) and because its disappearance rate in our system was much smaller than those of the other hydroxylated cholesterol derivatives (10), its postulation as an “intermediate” is unjustified.

Incubations in the presence of $^{13}$O$_2$ demonstrated that the oxygen atoms at C-20 and C-22 of the hydroxylated cholesterol derivatives derive from molecular oxygen. In two experiments, approximately 90% of the oxygen atmosphere was incorporated into these positions, whereas in the other two, 89% and 61% incorporation was found (cf. Table V). This suggested that a small variable amount of $^{18}$O$_2$ remained dissolved in the incubation media and was not completely equilibrated with the oxygen in the atmosphere. Further experiments (not presented here) indicated that more thorough evacuation of the reaction vessel results in better $^{18}$O incorporation.

The $^{13}$O content of the isolated pregnenolone was sometimes lower than that of the hydroxylated cholesterol derivatives, reflecting the $^{13}$O content of the atmosphere to the extent of 34 to 88%. This variability is most likely a result of $^{13}$O exchange during isolation, because reincubation of $[^{13}$O]pregnenolone did not result in a loss of label. Complete exchange of $^{13}$O with $^{18}$O resulted at pH 3 in water acidified with acetic acid. The use of large volumes of ethyl acetate in the isolation procedure was probably responsible for the relative loss of $^{13}$O in some experiments because strict control of the acid content of the solvent was not maintained. The lability of the C-20 carboxyl group was also encountered by other workers (16).

There was no significant difference between the $^{13}$O content of the (22R)-22-hydroxycholesterol and that of the glycol formed from cholesterol (cf. Table III). Only small differences between the $^{13}$O content of the oxygen atoms at C-20 and C-22 of the glycol were observed (cf. Table V).

Incubation of (22R)-22-hydroxycholesterol in an $^{18}$O-enriched atmosphere afforded (20R,22R)-20,22-dihydroxycholesterol with $^{18}$O at C-20. Within experimental error, no significant $^{18}$O was found at C-22. This is the first demonstration of the formation of the glycol form (22R)-22-hydroxycholesterol by mass spectrometry. The conversion of the monohydroxylated derivative to progesterone with rat adrenal mitochondrial preparations in the presence of $^{18}$O$_2$ has been demonstrated by mass spectrometry (16, 17).

In the incubations of cholesterol with $^{18}$O$_2$ and $^{18}$O$_2$ (devoid of $^{18}$O) the appearance of ions of m/e 291 (containing both $^{18}$O and $^{18}$O) in the spectra of the (trimethylsilyl) ether of the glycol clearly showed that the oxygen atoms of the side chain of the glycol derive from 2 separate oxygen molecules. As mentioned above, there was no significant difference

<table>
<thead>
<tr>
<th>Compound</th>
<th>m/e</th>
<th>Measured abundance</th>
<th>Corrected abundance</th>
<th>$^{13}$O Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>(20R,22R)-20,22-Dihydroxycholesterol (trimethylsilyl)</td>
<td>289</td>
<td>0.473</td>
<td>0.497</td>
<td></td>
</tr>
<tr>
<td></td>
<td>291</td>
<td>0.526</td>
<td>0.503</td>
<td>0.252*</td>
</tr>
<tr>
<td></td>
<td>461</td>
<td>0.482</td>
<td>0.519</td>
<td></td>
</tr>
<tr>
<td></td>
<td>463</td>
<td>0.518</td>
<td>0.481</td>
<td>0.481</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>316</td>
<td>0.510</td>
<td>0.518</td>
<td></td>
</tr>
<tr>
<td></td>
<td>318</td>
<td>0.490</td>
<td>0.482</td>
<td>0.482</td>
</tr>
</tbody>
</table>

* Represents the mean $^{13}$O content of the oxygen of the side chain.

<table>
<thead>
<tr>
<th>Incubation with cholesterol</th>
<th>$^{13}$O Content at positions</th>
<th>C-22</th>
<th>C-20</th>
<th>C-22</th>
<th>C-20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>of &quot;22R&quot;</td>
<td>of &quot;20,22R&quot;</td>
<td>of &quot;Pregn.&quot;</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.89</td>
<td>0.89</td>
<td>0.91</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.87</td>
<td>0.84</td>
<td>0.95</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.69</td>
<td>0.65</td>
<td>0.72</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>0.61</td>
<td>0.62</td>
<td>0.64</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.88</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.88</td>
<td>0.04</td>
<td>0.88</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* These were additional incubations with cholesterol done in a fashion similar to that described for C to F.

<table>
<thead>
<tr>
<th>Incubation with &quot;22R&quot;</th>
<th>C-20</th>
<th>C-22</th>
<th>C-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>0.88</td>
<td>0.04</td>
<td>0.88</td>
</tr>
</tbody>
</table>
between the $^{18}$O contents of the (22R)-22-hydroxycholesterol and the glycol. Their $^{18}$O contents were lower than those of the atmospheres but they most likely reflect the isotopic composition of the oxygen available to the enzyme. For this reason it was appropriate to ascertain the random nature of incorporation of oxygen atoms into the glycol by comparing the relative abundances of ions of $m/z$ 289, 291, and 293 with the binomial distribution obtained from the $^{18}$O content (a) of the side chain (cf. Table III). The data from incubations D and E (12) and F reflected the calculated binomial distribution $[(1-a)^{2a}(1-a:a)^2]$ to the extent of more than 95%, and are summarized in Table VI, in which the expected distribution for the "direct" insertion of the oxygen molecules is also given. Although the migration of trimethylsilyl groups between adjacent oxygen atoms both during silylation and mass spectrometric fragmentation has been observed (18), there is no evidence for interchange of oxygen atoms that are covalently bound to carbon. The formation, in the incubation with (22R)-22-hydroxycholesterol, of the glycol with $^{18}$O only at C-20, serves as evidence that there is no interchange of the oxygen atoms between C-20 and C-22 during the incubation, the silylation, the gas chromatography, or the mass spectrometry and that the random incorporation of the oxygen atoms into the glycol formed from cholesterol was the result of an enzymatic process.

**Table VI**

Experimental and expected distribution of isotopic oxygen in side chain of glycol formed from cholesterol incubated in presence of $^{18}$O and $^{18}$O$_2$ (without $^{18}$O$^{18}$O$^2$)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Isotopic species in side chain</th>
<th>$^{18}$O</th>
<th>$^{18}$O$^{18}$O$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>Expected binomial</td>
<td>0.312</td>
<td>0.474</td>
</tr>
<tr>
<td></td>
<td>Expected &quot;direct&quot; insertion</td>
<td>0.540</td>
<td>0.451</td>
</tr>
<tr>
<td>E</td>
<td>Expected binomial</td>
<td>0.474</td>
<td>0.418</td>
</tr>
<tr>
<td></td>
<td>Expected &quot;direct&quot; insertion</td>
<td>0.491</td>
<td>0.420</td>
</tr>
<tr>
<td>F</td>
<td>Expected binomial</td>
<td>0.502</td>
<td>0.399</td>
</tr>
<tr>
<td></td>
<td>Expected &quot;direct&quot; insertion</td>
<td>0.700</td>
<td>0.300</td>
</tr>
</tbody>
</table>

These results preclude an intramolecular rearrangement of a hydroperoxide (6, 7) as an intermediate step in the formation of the (20R,22R)-20,22-dihydroxycholesterol from cholesterol, and are consistent with its consecutive formation from cholesterol via the (22R)-22-hydroxycholesterol.

**Acknowledgment**—The technical assistance of Grace Han is gratefully acknowledged.

**REFERENCES**

Mass spectrometric study of the enzymatic conversion of cholesterol to (22R)-22-hydroxycholesterol, (20R,22R)-20,22-dihydroxycholesterol, and pregnenolone, and of (22R)-22-hydroxycholesterol to the lglycol and pregnenolone in bovine adrenocortical preparations. Mode of oxygen incorporation.

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