ENZYMATIC FORMATION OF (20R,22R)-20,22-DIHYDROXYCHOLESTEROL FROM CHOLESTEROL AND A MIXTURE OF $^{16}O_2$ AND $^{18}O_2$: RANDOM INCORPORATION OF OXYGEN ATOMS*

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SUMMARY

[4-^{14}C]Cholesterol was incubated with an adrenocortical preparation in the presence of $^{16}O_2$ and $^{18}O_2$ devoid of significant $^{16}O^{18}O$. Isolated (20R,22R)-20,22-dihydroxycholesterol was converted to a trimethylsilyl derivative and analyzed by gas chromatography - mass spectrometry to determine the isotope distribution of the oxygen atoms at C-20 and C-22. The ions of *m/e* 289, 291, and 293 (comprising the C_8 C-20 to C-27 side-chain and containing, respectively, $^{16}O_2$, $^{16}O^{18}O$, and $^{18}O_2$) exhibited a binomial distribution indicating that the oxygen atoms of the vicinal glycol were drawn at random from the atomic pool of the oxygen molecules. If both side-chain hydroxyl groups had originated from the atoms of the same oxygen molecule, the ion of *m/e* 291 would have been absent.

Previous analysis of kinetic data has indicated that (20R,22R)-20,22-dihydroxycholesterol, an intermediate in the enzymatic conversion of cholesterol to pregnenolone, may arise "directly" from cholesterol and not *via* (20S)-20-hydroxycholesterol or (22R)-22-hydroxycholesterol (1,2). This apparent one-step transformation could occur by insertion of a whole molecule of oxygen, possibly through the intermediacy of a hydroperoxide as suggested by Van Lier and Smith (3,4), or by a sequential hydroxylation on an organized multienzyme complex in which the monohydroxylated intermediates are not released into the medium (5), possibly involving transient free-radical or ionic species (6). However, no experimental data exist

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which would allow a distinction between the two types of mechanism of oxygen insertion into the side-chain: (a) derivation from the same oxygen molecule, or (b) derivation from separate molecules (drawing at random on the available atomic pool). This question could be resolved by incubating cholesterol in the presence of a mixture of $^{16}O_2$ and $^{18}O_2$ (devoid of $^{16}O^{18}O$). The mass spectrum of the fully silylated TMS derivative of the vicinal glycol contains an ion of *m/e* 289 which comprises the C_8 C-20 to C-27 side-chain containing the two oxygens at C-20 and C-22. If the oxygens originate from the same molecule, ions of *m/e* 289 and *m/e* 293 (containing, respectively, $^{16}O_2$ and $^{18}O_2$ in an intensity ratio equal to that present in the gas) would be observed. If the two oxygen atoms derive from different oxygen molecules an ion of *m/e* 291 (containing $^{16}O^{18}O$) would also be observed and the relative abundances of the ions of *m/e* 289, 291, and 293 would be in a binomial distribution, reflecting the isotopic content of the oxygen gas.

In this report we present mass spectral data on the isotopic distribution of oxygen in isolated (20R,22R)-20,22-dihydroxycholesterol after incubation of [4-^{14}C]cholesterol with a bovine adrenocortical mitochondrial acetone-dried powder preparation in the presence of $^{18}O_2$ and $^{16}O_2$.

EXPERIMENTAL PROCEDURE

A bovine adrenocortical mitochondrial acetone-dried powder was prepared as previously described (7). In each of the two experiments described here (A and B) 800 mg of the powder was homogenized in 88 ml of ice-cold 0.02 M potassium phosphate buffer (pH 7.3) (which was used throughout) and centrifuged at 30,000 x *g* for 1 hour in the cold room. The supernatant was used the same day (exp. B) or after storage at -20° for 20 hours (exp. A). The protein concentration of the supernatant, as determined by the Lowry reaction, was 2.2 mg/ml. Incubations were done at 24° in a 4 liter flask, the contents of which were stirred with a magnetic stirrer. 45 μCi of [4-^{14}C]cholesterol (790 μg), solubilized with 20 mg

Tween 80 in 1,120 ml buffer was preincubated for approximately 20 min with 35 ml (exp. A) or 40 ml (exp. B) of supernatant. During the preincubation the reaction vessel and the attached addition flask containing the TPNH generating system were evacuated and flushed with nitrogen several times to remove $^{16}O_2$. The residual vapor phase oxygen concentration, measured by injecting 1 ml into the heated inlet of the LKB 9000 mass spectrometer was <0.2%. 100 ml each of $^{16}O_2$ and $^{18}O_2$ (99.9%) were introduced to the evacuated vessel and the reaction was started by the addition of the TPNH generating system which contained 112 mg TPN, 1.34 g glucose-6-phosphate and 5 mg (exp. A) or 2.5 mg (exp. B) of yeast glucose-6-phosphate dehydrogenase dissolved in 35 ml of buffer. The reaction vessel was filled with nitrogen to atmospheric pressure and the incubation allowed to proceed for 20 min. The $^{16}O_2$:$^{18}O_2$ content of the vapor phase was monitored every two minutes during the reaction and was found to remain constant with no formation of $^{16}O^{18}O$. The total oxygen concentration was approximately 6%. The reaction was terminated by adding 2 liters of redistilled ethyl acetate which had been flushed with nitrogen for approximately 20 min. To the ethyl acetate extract tracer [1,2-$^3H_2$](20*R*,22*R*)-20,22-dihydroxycholesterol (1-2 ng) was added and the glycol isolated by partition chromatography on Celite 545 in several systems sufficient to achieve radiochemical purity as checked by the correspondence of the $^{14}C/^3H$ ratios as previously described (7). In addition to experiments A and B, a blank experiment with [4-^{14}C]cholesterol, TPNH and $^{16}O_2$ but containing 40 ml buffer instead of adrenal enzyme was also done precisely as described for experiment B and alongside it.

The isolated glycol was converted to a tri-TMS derivative with trimethylsilylimidazole and examined by GC-MS using an LKB 9000 instrument under the following conditions: flash heater, 270°; column, 6 ft 1% OV-1 on Gas Chrom Q (100-120 mesh) at 250°; electron energy, 22.5 eV. Partial mass spectra (*m/e* 270-310) were obtained by repetitive scanning as the

glycol TMS derivative emerged from the GC column (15 min). Relative abundances of ions of *m/e* 289, 291, and 293 were determined by calculating mean values obtained from ten successive mass spectral scans taken at the summit of the GC peak.

RESULTS AND DISCUSSION

The yield of the formed (20*R*,22*R*)-20,22-dihydroxycholesterol as determined from its ^{14}C content by liquid scintillation counting was approximately 600 ng in both experiments A and B. No significant radioactivity was found in the blank experiment. These results agreed with the intensities of the ions observed by mass spectrometry of the TMS derivatives; only background ions were seen in the blank experiment. That the isolated glycol arose almost exclusively from cholesterol and did not contain significant preformed material was also established in another experiment with the same adrenal enzyme preparation and [4-^{14}C]cholesterol except that only $^{16}O_2$ was used. In this experiment (to be described elsewhere) it was shown by mass spectrometry that the ^{12}C to ^{14}C ratio of the glycol and that of the added or reisolated cholesterol from the incubation were not significantly different.

The side-chain C_8 ion intensity distribution giving the abundance of the glycol TMS species containing $^{16}O_2$ (*m/e* 289), $^{16}O^{18}O$ (*m/e* 291), and $^{18}O_2$ (*m/e* 293) is given in Table I. Included in this Table is the oxygen composition of the atmosphere during the incubation as well as the calculated ion abundances for the two situations in which the two oxygen atoms of the glycol are derived either from two separate molecules or from the same molecule of oxygen. The ^{18}O content of the side-chain of the formed (20*R*,22*R*)-20,22-dihydroxycholesterol in experiments A and B was 90.5 and 68.0%, respectively, of that observed in the atmosphere during the incubation.

It is apparent from Table I that, whereas the oxygen atmosphere during the incubation had no significant $^{16}O^{18}O$ content, the recovered glycol con-

TABLE I

C_8-Side-chain fragment ion distribution in TMS derivative of (20R,22R)-20, 22 - dihydroxycholesterol formed from cholesterol incubated with an adrenocortical preparation in the presence of a mixture of $^{16}O_2$ and $^{18}O_2$.[a]

	Experiment A			Experiment B		
m/e	289	291	293	289	291	293
		%			%	
1. Measured ion abundance	29.3	46.9	23.7	44.2	42.5	13.2
2. Side-chain oxygen distribution	31.4[b]	47.4	21.2	47.7	41.7	10.6
3. Expected ion abundance: oxygen atoms from separate molecules	30.4[c]	49.5	20.1	47.0	43.1	9.9
	25.5[d]	50.1	24.6	28.8	49.7	21.4
4. Expected ion abundance: oxygen atoms from the same molecule	55.1[e]	0	44.9	68.5	0	31.5
	49.9[f]	1.1	49.0	53.7	0	46.3

a The C_8 fragment ions *m/e* 289, 291, and 293 contain $^{16}O_2$, $^{16}O^{18}O$, and $^{18}O_2$, respectively. The oxygen composition of the atmosphere during the incubation is given in row 4[f], where the *m/e* values stand for the oxygen species mentioned above.

b Contributions of naturally occurring heavy isotopes have been subtracted from the measured ion abundances.

c Binomial distribution ($a^2 : 2ab : b^2$) calculated from the oxygen isotope composition of the side-chain (given in 4[e]), where a=0.551 and b=0.449 (exp. A); a=0.685, b=0.315 (exp. B); a=^{16}O, b=^{18}O.

d Binomial distribution calculated from isotope content of the atmosphere (4[f]); a=0.505, b=0.496 (exp. A); a=0.537, b=0.463 (exp. B).

e Calculated from row 2.

f From the oxygen composition of the atmosphere during the incubation, which is given here.

tained molecules with both ^{16}O and ^{18}O in the side-chain (*m/e* 291). This result proves that a mechanism is operative in which the glycol arises from the attack of *separate* oxygen molecules. Had the glycol originated only through the attack of single oxygen molecules, the atoms of which would be incorporated as a unit, the ion at *m/e* 291 would have been absent. The

expected ion abundances for the single (whole) molecule incorporation are presented in rows 4^e and 4^f of Table I and are clearly different from the observations (row 2).

If each of the oxygen atoms of the glycol arose *only* through a random process by the attack of separate oxygen molecules, a binomial distribution of the ion abundances at *m/e* 289, 291, and 293 would be expected. Table I shows the expected distribution from the ^{18}O content of the C_8 side-chain ions (row 3^c) and from that in the vapor phase during the incubation (row 3^d). Although the calculated binomial distribution from the vapor phase isotope content of experiment A corresponded fairly well with the experimental results, in experiment B the observed ion abundance was biased towards the $^{16}O_2$ containing ion (compare row 2 with 3^d). Because the isotopic oxygen content of the atmosphere may not always accurately reflect the situation at the reaction site it is more valid to compare the observed frequencies with a binomial distribution calculated from the isotopic content of the side-chain itself. It is noteworthy (compare rows 2 and 3^c) that the isotope ratios in the side-chain ion conformed exceedingly well to a binomial distribution in both experiments A and B. The results indicated that at least 97% of the glycol arose by a random selection from the available oxygen atom pool and that no significant direct insertion of individual molecules occurs such as would be obtained if the glycol arose from an intramolecular rearrangement of a hydroperoxide.

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