limited accuracy of the potentiometric method, the best visual fit was made to first-order plots and standard deviations were calculated from the deviations of the experimental points from the lines.

With the very slowly reacting chlorides, complications were introduced by the reaction of the liberated hydrochloric acid with the ethanolic medium, particularly in the later stages. This was particularly true with *syn*-7-chloronorbornene (II) and 7-chloronorbornane (I) which gave complicated rate curves in consequence of the substantial contamination with nortricyclyl chloride, the reaction between ethanol and hydrochloric acid and the very low reactivities of the chlorides themselves. Figure 1 shows a typical plot of $-\log (a - x)$ against time. The steep initial slope results from a relatively rapid initial liberation of hydrochloric acid by the contaminating nortricyclyl chloride. When the concentration of nortricyclyl chloride becomes small, the hydrochloric acid concentration passes through a maximum because the rate of reaction of the acid with ethanol is faster than the solvolysis of II. With approximate reaction rates obtained for hydrochloric acid with ethanol under the experimental conditions at three separate initial acid concentrations comparable to those calculated to be present at the maximum in Fig. 1, it was possible to make a rough empirical correction of the rate data and obtain upper limits for the rate constants of II. A similar procedure was followed for I.

Products from the Solvolyses of *syn*- and *anti*-7-Chloro-*exo*-norbornyl *p*-Toluenesulfonates (IV and V) in Acetic Acid.—Two 25-ml. aliquots of a 0.0322 *M* solution of *syn*-7-chloro-*exo*-norbornyl *p*-toluenesulfonate (IV) in dry acetic acid containing 0.0308 *M* potassium acetate were heated at 78.2° for 45 minutes and 6 hours, respectively. Titration with perchloric acid showed that acetolysis had occurred in the two solutions to the extent of 10 and 70%, respectively. The reaction mixtures were diluted with water, neutralized with sodium carbonate and extracted with ether. The ether extracts were dried over sodium sulfate, evaporated to dryness and the chloronorbornyl acetates removed by trituration with cold pentane. The infrared spectra of the residues showed only IV to be present in the material from the 45-min. reaction while that from the 6-hour reaction showed both IV and V to be present, approximately in the ratio of 4:1.

Identical experiments starting with *anti*-7-chloro-*exo*-norbornyl *p*-toluenesulfonate (V) showed no significant rearrangement after 10% reaction and afforded about a 1:5 mixture of IV and V after 70% acetolysis.

A solution of 2.0 g. (0.0066 mole) of the sulfonate ester IV and 1.0 g. (0.010 mole) of potassium acetate in 50 ml. of dry acetic acid was heated for 24 hours at 100°. The mixture was cooled, the acetic acid neutralized with aqueous sodium carbonate solution and the products extracted with ether. The ether extract was dried over sodium sulfate, the ether evaporated and the residue distilled through a semi-micro column. A mixture of chloronorbornyl acetates (0.81 g.) was obtained, b.p. 70–80° (4 mm.), $n^{25}D$ 1.4808.

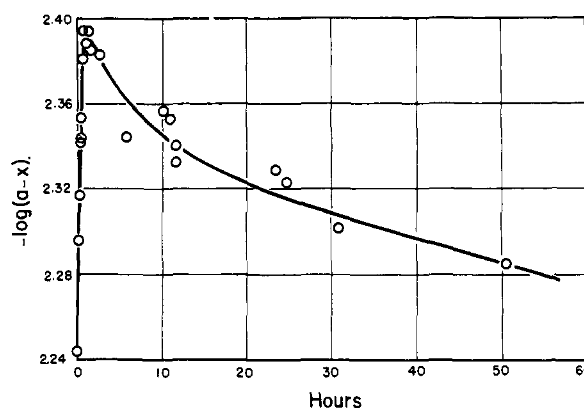

Fig. 1.—Solvolysis rate curve for *syn*-7-chloronorbornene in 50% ethanol at 99.7°.

Anal. Calcd. for $C_9H_{13}O_2Cl$: C, 57.29; H, 6.94; Cl, 18.80. Found: C, 57.57; H, 6.92; Cl, 18.47.

Sulfonate ester V was carried through the same procedure and afforded 0.82 g. of chloronorbornyl acetate mixture, b.p. 80–88° (4–5 mm.), $n^{25}D$ 1.4811.

Anal. Calcd. for $C_9H_{13}O_2Cl$: C, 57.29; H, 6.94; Cl, 18.80. Found: C, 57.31; H, 6.82; Cl, 18.75.

Infrared spectra of the two chloronorbornyl acetate mixtures from IV and V were very similar. Lithium aluminum hydride reduction of each chloronorbornyl acetate mixture yielded mixtures of colorless, waxy solids which were purified by short-path distillation at 128° (4 mm.). Comparison of the infrared spectra of the reduction products with those of synthetic mixtures of chlorohydrins VI and VII revealed that each was approximately a 1:1 mixture of the two chloronorborneols.

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Radiation Induced Oxidation of Cholesterol[1]

BY WILLIAM G. DAUBEN AND PIERRE H. PAYOT[2]

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When C^{14}-labeled cholesterol is stored in the presence of air, it is oxidized on and about the 5,6-double bond and, in the main, the epimeric 7-hydroxy, the 7-keto and the $5\alpha,6\beta$-dihydroxy derivatives are formed. Such an oxidation reaction requires both radiation and oxygen since unlabeled cholesterol in air or C^{14}-labeled cholesterol *in vacuo* are stable.

In 1953,[3] attention was drawn to the fact that certain C^{14}-labeled organic compounds underwent self-induced radiation damage and, subsequently,[4] this reaction has been studied in detail. At that time it also was reported that Chaikoff and his co-workers, in the Department of Physiology of the University of California, had found that C^{14}-labeled cholesterol possessing a specific activity of ~6.5 μc./mg. underwent decomposition over a period of approximately 18 months to the extent of 40%. The extremely large amount of transformation of this important sterol was unexpected and it was of importance to identify the products formed and to evaluate the role of the radiation in the transformation process.

The first samples of C^{14}-cholesterol investigated had been stored in a screw-cap vial and had been exposed to the atmosphere at frequent inter-

(1) This work was supported, in part, by the University of California Cancer Fund.

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vals in the 18 months period. Some preliminary experiments[5] indicated that rather than the molecular fission found in compounds such as choline, oxidation of the sterol had occurred. For example, it was found that the aged cholesterol possessed a band in the infrared at 5.9–6.0 μ, characteristic of a carbonyl group, and that the intensity of this band decreased after the material had been treated with Girard reagent. Isotopic dilution experiments showed that the carbonyl-containing material was not cholestenone. Furthermore, not all of the decomposition products could be simply ketonic derivatives of cholesterol since approximately 40% of the radioactivity would not precipitate with digitonin and the presence of a carbonyl group at C_6, C_7 or C_{20} does not interfere with this specific precipitation reaction.[6]

Ever since Schulze and Winterstein[7] showed that cholesterol upon exposure to air and sunlight underwent decomposition, there has been discussion as to the stability of cholesterol.[8] Thus, this present study has been concerned with three points: first, the exact nature of the compounds formed in the C^{14}-cholesterol; second, the role of air and solvents; and third, the role of radiation.

With regard to the chemical changes induced, from what is known about the oxidation of cholesterol or its esters by mild oxidizing agent such as manganese dioxide in petroleum ether[9] or oxygen in the presence of a metal catalyst,[10] the oxidation products of cholesterol would be expected to have oxygen functions at positions 5, 6 or 7. Furthermore, when cholesterol is irradiated in solid form with ultraviolet[11] light or in a polar solvent with X-rays,[12] the only products are compounds with oxygen functions at positions 5, 6 or 7. It is of interest to note that when cholesterol in a non-polar solvent is irradiated with X-rays,[13] the only isolable compound is the starting cholesterol.

In the present study, the amount of aged C^{14}-cholesterol was too small to permit direct isolation of the various products and so the mixture was analyzed using the isotopic dilution technique. The following oxidation products of cholesterol were measured: 7-ketocholesterol, 7α- and 7β-hydroxycholesterol, cholestane-$3\beta,5\alpha,6\beta$-triol and $3\beta,5\alpha$-diol-6-one. It was found that crystalline cholesterol labeled with C^{14} in the C_4-, C_{24}- or C_{26}-positions and stored in a manner so that it had contact with air, underwent decomposition, the amount of which depended upon both the time and the specific activity. All of the above mentioned oxygenated cholesterol derivatives were formed. For example, a sample of cholesterol-26-C^{14} which was approximately 24 months old had decomposed to the extent of 44% and the oxygenated products accounted for 40% of this change. The product composition was approximately 58% cholesterol, 11% 7-ketocholesterol, 14% 7α-hydroxycholesterol, 10% 7β-hydroxycholesterol, 5% cholestane-3β,-$5\alpha,6\beta$-triol and 2% cholestane-$3\beta,5\alpha$-diol-6-one. For comparison, it has been found that X-ray irradiation yields mostly the triol[12] while metal-catalyzed oxidation gives mostly 7-oxygenated derivatives.[10] The position of the label made no noticeable difference in the composition of the decomposition products.

Such a radiation-induced oxidation requires a source of oxygen either from atmospheric oxygen or water. To investigate whether other changes would occur due to the radiation alone, a highly dried and sublimed sample of cholesterol-4-C^{14} was stored *in vacuo* for a period of 2.5 months and no change was observed. A similar storage period in air brought about a change of 8% of the cholesterol. This stability of solid cholesterol *in vacuo* toward radiation also has been reported when the radiation is either 2–4 Mv. e^- or 1.3 Mev. γ (Co^{60}).[14]

To evaluate the autoxidation of cholesterol in the absence of radiation, unlabeled cholesterol was stored under the same conditions in which the labeled substance underwent decomposition. It was found that after a period of one year, although a slight yellow color had developed, the infrared spectrum of cholesterol had not changed. This finding clearly established the fact that cholesterol itself when stored in the presence of air but in the absence of ultraviolet light is quite stable and that the presence of the C^{14} is essential for the facile oxidation of the solid material.

Thus, it can be concluded that cholesterol is stable in air when stored in the absence of ultraviolet light and is stable toward radiation when stored *in vacuo*. However, when cholesterol is in the presence of oxygen-containing compounds, such as air and water, it is very susceptible to radiation and undergoes oxidation on and about the 5,6-double bond. From a consideration of the extent of this radiation-induced oxidation and the amount of radiation available from the C^{14} present, it must be concluded that the reaction is of the chain type.

This establishment of a radiation sensitivity of cholesterol makes it imperative for workers utilizing C^{14}-labeled material to exert great care in its storage and to establish the past history of the sample. Since the 7-hydroxycholesterols, the major products of the oxidation reaction, are of the fast reaction type in the Liebermann–Burchard test as modified by Baumann, Moore and Idler,[15] this color test allows one to measure rapidly the purity of the labeled cholesterol sample.

These present findings of radiation-induced oxidation of cholesterol are of special interest in view of

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the recent report of Fieser and his collaborators[16] that 6β-hydroperoxycholest-4-ene-3-one is a carcinogenic compound. It is well-known that radiation can induce tumor formation and since cholesterol, a normal body constituent, can be oxygenated at C_6 in the presence of radiation, it is suggestive that perhaps cholesterol is the link between irradiation and tumor initiation.

Experimental[17]

Preparation of Steroids. (a) Unlabeled Materials.—7-Ketocholesterol was prepared from cholesteryl acetate by oxidation with *t*-butyl chromate[18] followed by hydrolysis with potassium carbonate.[19] 7β-Hydroxycholesterol was obtained by $LiAlH_4$ reduction of 7-ketocholesteryl acetate[20] and purified *via* its benzoate.[20,21] 7α-Hydroxycholesterol was prepared from the 7β-epimer by acid-catalyzed equilibration.[22] Commercial cholesterol was purified by bromination and debromination with zinc.[23] Cholestane-3β,5α,6β-triol was prepared by hydroxylation of cholesterol with hydrogen peroxide in formic acid[24] and subsequent oxidation of the triol with N-bromosuccinimide gave cholestane-3β,5α-diol-6-one.[24] It was found that the diolone prepared by this method was contaminated with some triol. This was detected by careful chromatography (see procedure below) on alumina and under our conditions, the diolone was eluted with $CHCl_3$ containing 1.0% MeOH and the triol was obtained with $CHCl_3$ containing 1.5% MeOH. Since the melting points of these two compounds lie close together and the reported values vary widely and, also, since there is little or no depression in a mixed melting point experiment, the identity of the fractions in the chromatogram was verified by forming the monoacetate of the diolone and the diacetate of the triol. The melting points found for the pure diolone and triol are 233–235° and 229–233°, respectively. Purification of the crude diolone also could be achieved by treatment of the material with bromine in aqueous ethanol.[25]

(b) Labeled Steroids.—Cholesterol-4-C^{14} and cholesterol-26-C^{14} were prepared as described earlier[26,27] and cholesterol-24-C^{14} was prepared by a modified procedure described elsewhere.[28] The labeled materials were purified via chromatography and dibromide formation before use.

Radioactivity Determinations.—All samples were oxidized under reduced pressure with the oxidation mixture of Van Slyke and Folch.[29] The carbon dioxide was collected in NaOH solution and the $BaCO_3$ precipitated and counted in the usual fashion.[30]

Infrared Spectra.—All spectra were taken in CS_2 solution at a concentration of 50 mg./ml. using a 0.1-mm. thick cell. In the case of the aged samples of cholesterol, in addition to the usual sharp band at 2.7 μ which is characteristic of the hydroxyl, a broad band appeared between 2.8–3.1 μ. The carbonyl band of the 7-ketocholesterol which appears at 6.0 μ could only be detected when the compound was present to the extent of more than 5%.

Chromatographic Analysis.—The technique of fractional elution of Reichstein and Shoppee[31] was followed, using an alumina which had been freed from alkali without using nitric acid and which had been reactivated at 180–190°.[32] All solvents were highly purified and the following sequence of solvents for elution was followed: pentane, petroleum ether (b.p. 40–50°) or hexane, benzene, ether, chloroform and methanol. Cholesterol was eluted with benzene–ether (5:2), 7-ketocholesterol with benzene–ether (1:1), 7α-hydroxycholesterol with ether–chloroform (1:1) and 7β-hydroxycholesterol with chloroform. The diolone and triol solvent sequence is described above.

General Pattern for the Analysis of Aged C^{14}-Cholesterol Using the Isotopic Dilution Technique.—Aged C^{14}-cholesterol (1 mg.) was mixed in chloroform with 49.5 mg. of unlabeled cholesterol (A) and 49.5 mg. of one of the cholesterol oxidation products (B). The solvent was evaporated under reduced pressure, the residue dried overnight in a high vacuum at 70° and then chromatographed on alumina. The chromatogram consisted generally of 30–40 fractions, from which compounds A and B were recovered. Several recrystallizations of each A and B were performed and then the specific activities were determined.

The last mother liquors of these crystallizations were freed from solvent and dried. The residues were acetylated with acetic anhydride and pyridine and the specific activities of the purified acetates determined. If the free sterol was difficult to purify,[33] the acetate mother liquors were hydrolyzed with KOH in methanol and the free sterol esterified with benzoyl chloride in methylene chloride containing a trace of pyridine. The excess benzoyl chloride was destroyed by allowing it to react with glycine and the purified steroidal benzoates analyzed.

Analyses of Aged C^{14}-Cholesterol Samples. (a) Cholesterol-26-C^{14}.—The sample had a specific activity of 3.4×10^5 cts./min./mg. C (1.53 μc./mg. cholesterol) and had been stored in a screw cap vial for a period of two years and had been exposed to the air at various intervals. The material analyzed as follows:

Compound	Total activity, %
A, Cholesterol	68
Cholesteryl acetate	66
B, 7-Ketocholesterol	12
7-Ketocholesteryl acetate	11
A, Cholesterol	66
Cholesteryl acetate	67
B, Cholestane-3β,5α,6β-triol	6
Cholestane-3β,5α,6β-triol-3,6-diacetate	5
A, Cholesterol	68
Cholesteryl acetate	66
B, Cholestane-3β,5α-diol-6-one	2
Cholestane-3β,5α-diol-6-one-3-acetate	2
A, Cholesterol	66
Cholesteryl acetate	65
B, 7α-Hydroxycholesterol	..[a]
7β-Hydroxycholesterol-3,7-diacetate	9
7β-Hydroxycholesterol-3,7-dibenzoate	10
A, Cholesterol	66
Cholesteryl acetate	65
B, 7α-Hydroxycholesterol	..[a]
7α-Hydroxycholesterol-3,7-diacetate	14
7α-Hydroxycholesterol-3,7-dibenzoate	11

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(b) **Cholesterol-24-C[14].**—Four separate experiments were performed. 1. A freshly prepared sample (m.p. 145–146°, s.a. 0.18 μc./mg. cholesterol) was analyzed by the above described procedure for cholesterol and 7-ketocholesterol. Within the limits of experimental error, the entire activity resided in the cholesterol fraction; a trace (<1%) was found in the 7-ketocholesterol fraction.

2. A portion of the same sample (m.p. 145–146°) was first dried in a high vacuum at 70° for 12 hours and then sealed in a vial and stored in the dark for 15 months. After this period of time, the m.p. had dropped to 129–130° and the infrared spectrum showed the broad band in the 2.8–3.1 μ region, characteristic of the decomposition products. The material was analyzed for cholesterol and 7-ketocholesterol. The cholesterol was found to possess 93% of the total activity and the 7-ketocholesterol 3%.

3. A portion of the same sample was kept for a month in a screw cap vial filled with air and then sealed into a vial under reduced pressure and stored for 14 months in the darkness. The aged material sintered 120° and melted at 129–130°. The infrared spectrum showed the broad band at 2.8–3.1 μ and a questionable indication of a carbonyl band. The sample was analyzed for cholesterol and 7-ketocholesterol and was found to have 87% of the total activity in the former and 7% of the total activity in the latter.

4. A portion of the sample was stored in a screw cap vial and the vial was opened at least once a week for 15 months and the sterol stirred with a glass rod. After this period, the m.p. was 126–127° and the infrared spectrum showed the broad band at 2.8–3.1 μ. The sample was analyzed for only three components and the results were: 83% cholesterol, 5% 7-ketocholesterol and 5% 7β-hydroxycholesterol.

(c) **Cholesterol-4-C[14].**—Two separate experiments were performed. 1. A sample of freshly prepared cholesterol (m.p. 146–147°, s.a. 3.83 μc./mg. cholesterol) was stored in a screw cap vial filled with air for a period of three months. After this period the m.p. was 129–130° and the infrared spectrum showed the broad band at 2.8–3.1 μ and a weak band at 6.0 μ. The sample was only analyzed for cholesterol and 7-ketocholesterol and these compounds possessed 92 and 7%, respectively, of the total activity.

2. A portion of the same sample was sublimed in a high vacuum and sealed directly in a vial without changing the pressure. The material was stored for 10 weeks in the darkness. At the end of this period, the material had a m.p. of 146–147° and within experimental error, all the activity was found to reside in the cholesterol when the sample was analyzed.

Experiments with Unlabeled Cholesterol. (a).—Purified cholesterol (m.p. 146–147°) was sealed in an evacuated vial and stored for one year in the darkness. At the end of this period the m.p. had not changed and the infrared spectrum was identical with that of the material at the start of the experiment.

(b).—Purified cholesterol (m.p. 146–147°) was stored for one year in a screw cap vial in the dark. At least once a week the vial was opened and the contents stirred with a glass rod. After one year, the cholesterol was still colorless but the crystals stuck together. After drying the sample, both the m.p. and the infrared spectrum were identical with that obtained at the start of the experiment.

(c).—Purified cholesterol (m.p. 146–147°) was stored for 18 months in a dark bottle equipped with a stirring device. The cholesterol was exposed to the atmosphere of the laboratory, only protected from the dust. The material was stirred intermittently. At the end of the experiment, the cholesterol was yellowish but the m.p., rotation and the infrared spectrum were identical with that obtained at the start of the experiment.

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[CONTRIBUTION FROM THE DIVISION OF CANCER RESEARCH, DEPARTMENT OF SURGERY, UNIVERSITY OF ROCHESTER MEDICAL CENTER]

The Preparation of Δ^5-Androsten-17β-ol-3,7-dione 17-Acetate 3-Ethylene Ketal and Some of its Reactions

BY P. N. RAO[1] AND P. KURATH

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After the oxidation of Δ^5-androsten-17β-ol-3-one 17-acetate 3-ethylene ketal with *t*-butyl chromate, the starting material and Δ^5-androsten-17β-ol-3,7-dione 17-acetate 3-ethylene ketal were isolated from the reaction mixture. Several reactions carried out with the latter proved the assigned 7-ketone structure.

In view of a recent publication by Lenhard and Bernstein[2] describing a method for the preparation of 7-keto derivatives of ethylene ketals of Δ^4-3-ketosteroids, it seems desirable to report a different approach to the synthesis of such compounds.

After the oxidation of Δ^5-androsten-17β-ol-3-one 17-acetate 3-ethylene ketal (I)[3] with *t*-butyl chromate, the starting material I and an α,β-unsaturated ketone II with a high ultraviolet absorption maximum (log ε 4.1) at 241 mμ were isolated. The high absorption maximum of II indicated that the oxidation of I occurred on the allylic center of C-7 rather than of C-4[4] and the new product was, therefore, formulated as Δ^5-androsten-17β-ol-3,7-dione 17-acetate 3-ethylene ketal (II).

Following the alkaline hydrolysis of II, the $\Delta^{3,5}$-androstadiene-3-(β-hydroxy)-ethoxy-17β-ol-7-one (III) was isolated. Treatment of II with methanolic sulfuric acid, followed by alkaline hydrolysis to ensure complete saponification of the 17-acetoxy group, yielded $\Delta^{3,5}$-androstadiene-3-methoxy-17β-ol-7-one (IV) as the only crystalline product. This compound had the expected ultraviolet absorption maximum at 311 mμ, similar to the ultraviolet of III.[2,5]

These two hydrolysis experiments indicated that the formulation of II was correct. Oppenauer oxidation of the 17-hydroxy group in IV yielded the $\Delta^{3,5}$-androstadiene-3-methoxy-7,17-dione (V). Following a similar oxidation of the Δ^5-androstene-3β,17β-diol-7-one (VI),[6] the $\Delta^{3,5}$-androstadiene-3-ol-7,17-dione (VII) was obtained which, on treatment with methanolic sulfuric acid, yielded a compound identical with V. Inasmuch as V was

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