Studies on the Biosynthesis of Cholesterol

4. DEGRADATION OF RINGS C AND D*

By J. W. CORNFORTH and IRENE YOUHOTSKY GORE
National Institute for Medical Research, Mill Hill, London

and G. POPJAK
M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, Ducane Road, London, W. 12

(*Received* 19 *June* 1956)

Chemical degradation of cholesterol biosynthesized from labelled acetic acid has been the subject of several communications from our own and other Laboratories [for review and bibliography see Cornforth (1954)]; the aim has been to discover which carbon atoms in cholesterol originate from the methyl group of acetic acid and which from the carboxyl group. The completion of this project is described in the present paper. When work was begun, the biosynthetic origin of C-7, C-8, C-9, C-11, C-12, C-14, C-15, C-16 and C-17 was unknown, and confirmation was desirable for C-13 and C-18. Later, the derivation of C-7 from the methyl group of acetate was proved by Bloch (1953) and by Dauben & Takemura (1953).

METHODS AND EXPERIMENTAL

Biosynthesis

[^{14}C]*Cholesterol.* Labelled cholesterol was obtained by incubation of liver slices of young rats with either [*Me*-^{14}C]acetate or [*carboxy*-^{14}C]acetate as described previously (Cornforth, Hunter & Popjak, 1953*a*). The unsaponifiable material extracted from the alkaline digest of the slices was first chromatographed on alumina (B.D.H. chromatographic grade) previously washed with methyl formate. Light petroleum (b.p. 40–60°), acetone–ethyl ether (1:1, v/v) and methanol were used for elution in succession. Digitonides were prepared from the acetone–ether eluate. The cholesterol, after decomposition of the digitonide by pyridine–ether, was purified through the dibromide and recrystallized from methanol. After preliminary dilution with inactive material, the cholesterol samples thus prepared were finally obtained for degradation as the acetates. Small samples of each were set aside as standards for assay for ^{14}C.

Derivatives of [^{14}C]cholesterol biosynthesized from $CH_3.{}^{14}CO_2H$ and $^{14}CH_3.CO_2H$ will be referred to as carboxyl-labelled and methyl-labelled respectively. Carboxyl-labelled cholesteryl acetate (2·324 g.) with a specific activity of 3·58 μc/g. and methyl-labelled cholesteryl acetate (2·538 g.) with a specific activity of 5·28 μc/g. served as starting materials for all degradations.

It might be mentioned that the [^{14}C]squalene biosynthesized from [*Me*-^{14}C]acetate, the complete degradation of which has been reported previously (Cornforth & Popjak, 1954), was obtained from these same biosynthetic experiments.

Manipulations before fission

In order to separate from each other the carbon atoms in ring D of cholesterol (I; R = H) it was necessary to open this ring. Our first task, achieved by modification of procedures already known, was the replacement of the existing double bond at 5:6 by a double bond at 14:15.

Bromination of cholesteryl esters with *N*-bromosuccinimide and dehydrobromination of the crude product containing 7-bromo compounds is an important method for preparing cholesta-5:7-dienes and several procedures have been published; we followed that of Bernstein, Binovi, Dorfman, Sax & Subbarow (1949). The isomeric 4:6-diene is always formed as a by-product of this reaction, and to secure a good recovery of pure 5:7-diene (II; R = Ac) would have been difficult. To the partially purified product from the [^{14}C]cholesterols, after saponification, a relatively large quantity of inactive cholesta-5:7-diene-3β-ol (II; R = H) was therefore added; radioactive diene (II; R = H) sufficiently pure for the next step was then obtained without trouble.

Partial hydrogenation of the diene to cholest-7-en-3β-ol (III; R = H) proceeded smoothly in benzene solution with Raney nickel as catalyst (Ruyle *et al.* 1952). Migration of the 7:8 double bond to the 8:14-position occurred on shaking III (R = H) with hydrogen and a platinum catalyst in acetic acid (cf. Wieland, Rath & Benend, 1941); the product, cholest-8(14)-en-3β-ol (IV; R = H) was formed almost quantitatively.

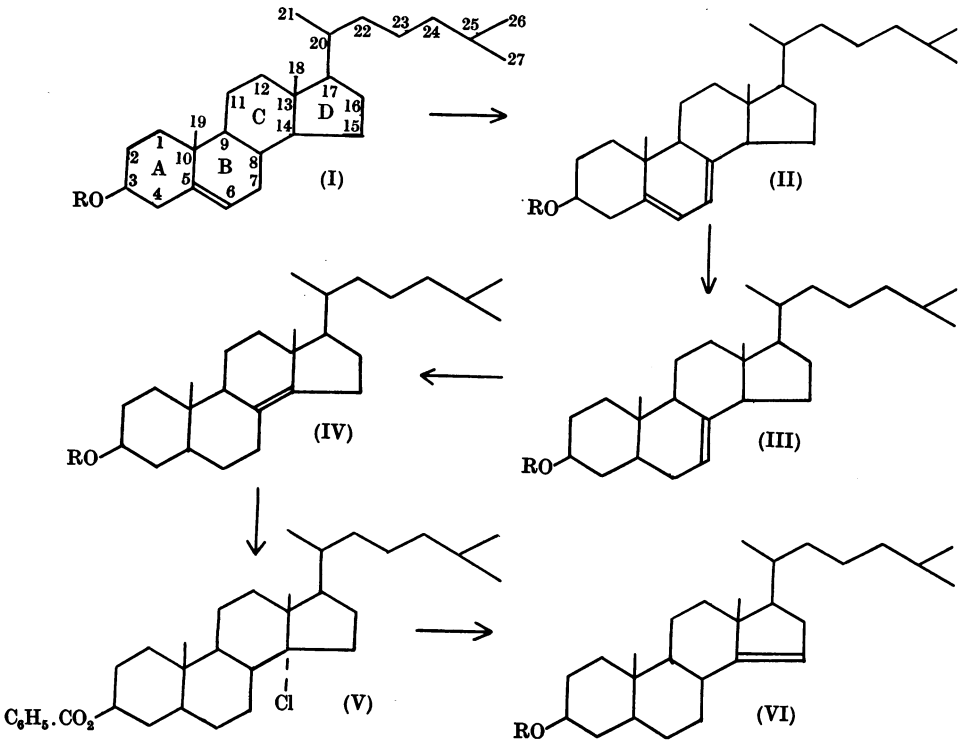
The free hydroxyl group in IV (R = H) was benzoylated before the next isomerization, as this facilitated isolation of the product. The original procedure of Schenck, Buchholz & Wiese (1936) for

* Part 3: Cornforth & Popjak (1954).

isomerization of this benzoate [IV; R = C_6H_5.CO] with hydrogen chloride in chloroform at 0° gave moderate yields even when more concentrated solutions were used (Heilbron & Wilkinson, 1932) and a milder method was sought. Addition of hydrogen chloride took place in chloroform solution at −30° and the adduct, presumably the 14α-chloro derivative (V), could if necessary be isolated. When the chloroform solution containing this adduct was shaken with aqueous $NaHCO_3$ dehydrochlorination occurred slowly, but it was accelerated on addition of ether. Cholest-14-en-3β-yl benzoate (VI; R = C_6H_5.CO) was then obtained in 75–80 % yield. By renewed treatment of the mother liquor containing the 8(14)-isomeride the yield was raised to 90 %.

Radioactive cholesta-5:7-dien-3β-ols. To carboxyl-labelled cholesteryl acetate (2·267 g.) in light petroleum [27·5 ml.; b.p. 66–67°; purified according to Bernstein *et al.* (1949)] was added finely powdered *N*-bromosuccinimide (1·152 g.) and the mixture was boiled under reflux for 12 min. in the light and heat from two Photoflood lamps (Royal Ediswan no. 1). Collidine (1·14 ml.; redistilled and kept over KOH) was added immediately before the lamps were switched off. Succinimide was removed by filtration and the solvent was evaporated at room temperature under reduced pressure. To the residue one half of a solution of collidine (0·46 ml.) in xylene (13·6 ml.) was added; evacuation was then resumed for a short time before adding the remainder. The mixture was boiled under N_2 at atmospheric pressure for 15 min. Collidine hydrobromide was removed by washing with water; the xylene solution was extracted successively with 70 ml. portions of 0·5 N-HCl, water, 0·5 M-$NaHCO_3$ and water. It was then dried ($MgSO_4$) and stirred with a little charcoal, filtered, and evaporated at low pressure (capillary fed with N_2). After removal of the remaining xylene in high vacuum the residue was dissolved in acetone–methanol (1:2, v/v; 7·5 ml.), seeded and left under N_2 at −5°. The solid was collected at −5° and washed with small amounts of 1:2 (v/v) and then 1:5 (v/v) acetone–methanol; it weighed 1·57 g. This material was boiled under N_2 for 45 min. with 16 ml. of methanolic KOH (5 %, w/v). Ether was added and the mixture was washed repeatedly with small quantities of ice-water. Non-radioactive cholesta-5:7-dien-3β-ol (7·5 g.) dissolved in ether was then added and the solution was concentrated under N_2 to 30–40 ml. When crystals appeared, warm methanol was added; the mixture was cooled to −5° and the solid was collected after 2 hr., washed with chilled methanol, and dried *in vacuo*. The yield was 7·90 g.; m.p. 142–145° (melting-points, except those taken on the Kofler block, were observed in open capillaries and are uncorrected).

Methyl-labelled cholesteryl acetate (2·524 g.) was treated in the same manner, but for some undiscovered reason the recovery of crude crystalline product was lower (0·949 g.). Since the ultraviolet absorption at 282 mμ. of the mother liquor indicated that much diene had remained in solution, the solid and the mother liquor were both saponified. To the product from the mother liquor, non-radioactive cholesta-5:7-dien-3β-ol (1·5 g.) was added. The mixture was dissolved in a minimum of boiling acetone and allowed to crystallize at −5°. The crystals after washing with chilled 2:1 (v/v)



acetone–methanol and drying weighed 1·46 g.; m.p. 136–141°. This was added along with 6 g. of non-radioactive diene to the product from saponification of the solid and the whole was recrystallized from acetone to give 6·866 g., m.p. 145–148°.

Radioactive cholest-7-en-3β-ols. To carboxyl-labelled cholesta-5:7-dien-3β-ol (7·7 g.) in benzene (200 ml.; thiophen-free; previously refluxed with Raney nickel), Raney nickel (approx. 2 ml. of a settled suspension in benzene) was added and H_2 was admitted at 50 lb./in.^{2}. After shaking for 2 hr. and leaving overnight a small amount of diene had remained unreduced, as shown by absorption at 282 mμ., and shaking was continued for 3 hr. The solution was filtered through kieselguhr and the solvent was removed under N_2 at low pressure. The residue was recrystallized from methanol (80 ml.) and dried *in vacuo*; 6·90 g., m.p. 124°. A second crop after recrystallization weighed 0·16 g.; m.p. 119–121°.

The methyl-labelled analogue was prepared in the same way from the corresponding diene (6·866 g.); yield 6·66 g., m.p. 122–123°. Optical rotations were not measured with these preparations but preliminary investigations with non-radioactive material gave a product m.p. 124°, $[\alpha]_D \pm 0°$. Schenck *et al.* (1936) gave m.p. 122–123°; $[\alpha]_D \pm 0°$. (Specific rotations refer to $CHCl_3$ solutions when the solvent is unspecified.)

Radioactive cholest-8(14)-en-3β-ols. Carboxyl-labelled cholest-7-en-3β-ol (7·06 g.) was triturated with acetic acid (80 ml. redistilled from CrO_3) and the suspension was added to Pt black (from 340 mg. of PtO_2) in acetic acid (80 ml.). The mixture was shaken for 2 hr. with H_2. Chloroform was added to dissolve the product; the catalyst was removed by filtration and the solvents by evaporation at low pressure under N_2. The residue was recrystallized from methanol to give 7·06 g., m.p. 121°. A second crop after recrystallization weighed 0·035 g.; m.p. 119°. The net gain in weight is attributable to solvation.

Methyl-labelled cholest-7-en-3β-ol (6·6 g.) was treated in the same manner to give 6·41 g., m.p. 121°.

The non-radioactive product had m.p. 120°; $[\alpha]_D^{24} + 21°$. Schenck *et al.* (1936) gave m.p. 119–120°; $[\alpha]_D^{21} + 20·36°$.

Radioactive cholest-8(14)-en-3β-yl benzoates. To carboxyl-labelled cholest-8(14)-en-3β-ol (7·095 g.) in pyridine (35 ml.) benzoyl chloride (4·7 ml.) was added with shaking. After 21 hr. the mixture was stirred with water (500 ml.) for 1 hr. and the solid was collected, washed with water and methanol, dried and recrystallized from acetone. It was later suspected that benzoylation was incomplete and the procedure was therefore repeated. This gave 5·72 g.; melting at 111–113° to a cloudy liquid which cleared at 138–140°; from crystallization of mother liquors a further 1·67 g. (m.p. 107–110°) and 0·42 g. (m.p. 108°) were obtained.

Methyl-labelled cholest-8(14)-en-3β-ol (6·4 g.) was twice benzoylated in similar fashion to give 6·04 g., m.p. 112–113° (clear at 140–141°), 0·70 g., m.p. 106–109° (clear at 133°) and 0·355 g., m.p. 112° (clear at 134°).

The non-radioactive product had m.p. 114° (clear at 144°), $[\alpha]_D^{21} + 11°$. Schenck *et al.* (1936) gave m.p. 115° (clear at 140°), $[\alpha]_D^{20} + 8·53°$.

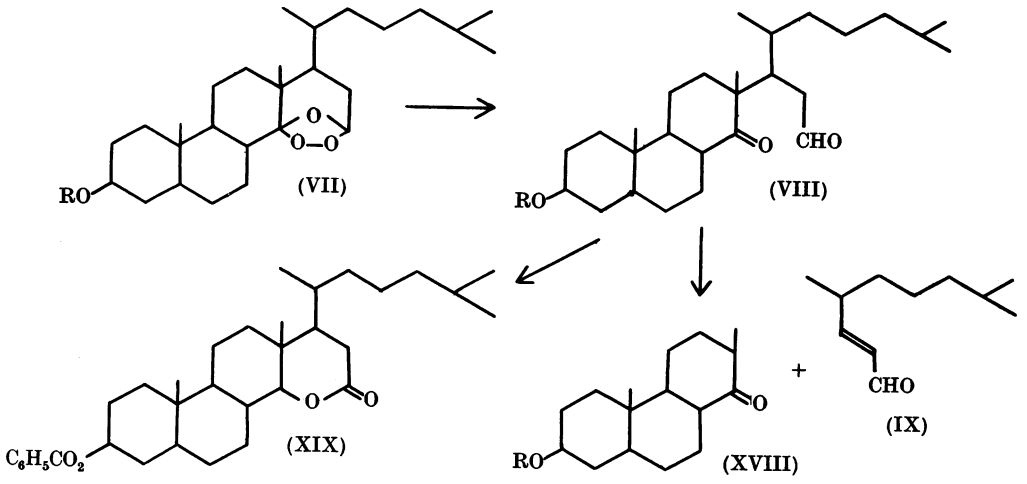
Radioactive cholest-14-en-3β-yl benzoates. Carboxyl-labelled cholest-8(14)-en-3β-yl benzoate (3·0 g.) was dissolved in $CHCl_3$ (6 ml.) and a gentle stream of dry HCl was passed for 2 hr. through the solution at −30°. The pressure in the reaction vessel was then lowered to about 20 mm. without interrupting the cooling; this procedure removed most of the HCl and some of the $CHCl_3$. The residue was poured into 0·5 M-$NaHCO_3$ (10 ml.), a little cold $CHCl_3$ being used for washing. Ether was then added; the mixture was left for ½ hr., shaken, and the aqueous layer run off. The product which had separated from solution was redissolved ($CHCl_3$) and the dried ($MgSO_4$) solution evaporated at low pressure. The residue was recrystallized as rapidly as possible from benzene (8 ml.) by addition of ethanol (16 ml.). The product (1·88 g.) crystallized in needles, m.p. 167°. A second run with 2·70 g. of the 8(14)-ene gave 1·735 g., m.p. 168·5°. The mother liquors from these two runs on further crystallization gave 0·895 g., m.p. 164·5–167°. The total from 5·72 g. was thus 4·51 g. or 79 %. The less pure 8(14)-ene from the previous stage (1·67 + 0·42 g.) was also isomerized, after being united with material (0·7 g., m.p. 111°) from the mother liquors of the first isomerizations, to give 2·01 g., m.p. 167–167·5°. Finally the combined mother liquors were evaporated and the residue was isomerized: this gave 0·435 g., m.p. 166°. Thus from a total of 7·79 g. of 8(14)-ene the yield was 6·955 g. (89 %) of a satisfactory product.

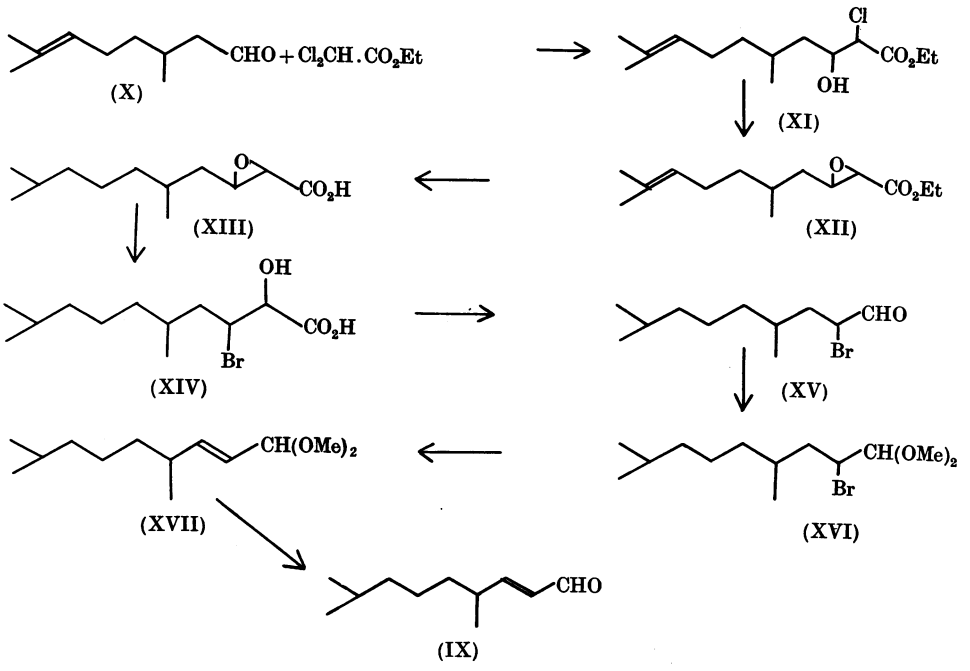
The methyl-labelled cholest-8(14)-en-3β-yl benzoate (6·04 + 0·70 + 0·355 g.) was isomerized in similar fashion to give a total of 6·005 g. (92 %), m.p. 165°. The highest m.p. observed with non-radioactive cholest-14-en-3β-yl benzoate was 168·5°; $[\alpha]_D^{22} + 31·5°$. Schenck *et al.* (1936) gave m.p. 168°; $[\alpha]_D^{22} + 32·54°$.

14α(?)-Chlorocholestan-3β-yl benzoate. Non-radioactive cholest-8(14)-enyl benzoate (1 g.) in $CHCl_3$ (3 ml.) was treated at −30° with HCl as above; after removal of excess of HCl the remaining solvent was evaporated at −30° in high vacuum. To the solid white residue was added light petroleum (b.p. 40–60°) cooled to −30°. The solid was collected quickly on a filter cooled by solid CO_2 and dried at −30° in high vacuum. The product after being kept overnight at −5° had m.p. 153–156° (clear at 158°). (Found: Cl, 6·7. $C_{34}H_{51}O_2Cl$ requires Cl, 6·7 %.) This substance (200 mg.) dissolved in ether (5 ml.) and $CHCl_3$ (2 ml.) was shaken with 0·5 M-$NaHCO_3$. The product on crystallization from benzene–ethanol gave cholest-14-en-3β-yl benzoate (0·13 g.), m.p. 166–166·5°.

Cleavage of ring D

The radioactive material was now ready for cleavage of ring D. Ozonolysis of the 14-ene (VI), reduction of the ozonide (VII) and pyrolysis of the resulting keto aldehyde (VIII) was the plan, for this sequence had been demonstrated with the ergostene analogues (Achtermann, 1934; Laucht, 1935). Cholest-14-en-3β-ol (VI; R = H), prepared by saponification of the benzoate (Schenck *et al.* 1936), was ozonized in hexane and the ozonide was reduced by zinc and acetic acid. After several trials we chose to pyrolyse the non-crystalline keto aldehyde (VIII) in *cyclo*hexane at 210–220°, having observed the appearance under these conditions of a substance volatile in steam and absorbing maximally at 222 mμ. These properties would be expected of the aldehyde (IX). A crystalline semicarbazone was in fact prepared from the steam-volatile material, and its composition indicated its origin from a C_{11} carbonyl compound.





The semicarbazone was optically active. Thereby, its synthesis from a product of known configuration—already desirable in order to prove its structure and to secure more material for experimental degradations—offered the opportunity of determining the absolute configuration of cholesterol. We therefore proceeded to synthesize the semicarbazone of the aldehyde (IX) from (+)-citronellal. The identity of the two specimens from cholesterol and from citronellal, and the stereochemical conclusions drawn from this identity, have already been reported (Cornforth, Youhotsky & Popjak, 1954); experimental details of the synthesis are now given. The transformation

$$R.CH_2.CHO \rightarrow R.CH{:}CH.CHO$$

might be effected by several methods; we chose one which seemed unlikely to cause racemization at any stage.

Condensation of (+)-citronellal (X) with ethyl dichloroacetate and magnesium amalgam (method of Darzens & Levy, 1937) gave the β-hydroxy-α-chloro ester (XI) which was converted by ethanolic sodium ethoxide into the glycidic ester (XII). The

glycide ester (XII) was saponified and the acidic product was hydrogenated in ethyl acetate over a platinum catalyst. The crude saturated glycidic acid (XIII) in benzene readily absorbed hydrogen bromide. The resulting α-hydroxy-β-bromo acid (XIV) was oxidized by lead tetraacetate in benzene. A neutral product consisting principally of the bromo-aldehyde (XV) was obtained. This was immediately converted into the dimethyl acetal (XVI) by reaction with methanol containing ethyl orthoformate and a little hydrogen chloride. This bromo-acetal was distilled but not further purified; it was boiled with methanolic KOH to produce the unsaturated acetal (XVII). Gentle hydrolysis with acid then produced a substance absorbing maximally at 222 mμ. and affording a crystalline semicarbazone identical with the semicarbazone from the product of pyrolysis (IX).

Ozonization of cholest-14-en-3β-ol. Cholest-14-en-3β-ol, m.p. 130–131°, was prepared from the benzoate by the procedure of Schenck *et al.* (1936). Ozonized oxygen was passed into a solution of this alcohol (2·5 g.) in hexane (250 ml.) at 0° until a blue colour persisted. An amorphous solid was precipitated. This was collected (2·025 g.) and shaken in acetic acid (60 ml.) with Zn powder (2·5 g.) for 45 min., the temperature rising to 40°. Water (140 ml.) and ether (100 ml.) were added, the Zn was removed and the ethereal layer after washing with water and 0·5 M-$NaHCO_3$ was dried ($MgSO_4$) and evaporated at low pressure. The glassy residue containing the keto aldehyde (VIII; R = H) weighed 1·5 g.

Pyrolysis of 3β-hydroxy-14-oxo-14:15-secocholestan-15-al. The keto aldehyde (302 mg.) in *cyclo*hexane (15 ml.) was sealed under N_2 in two tubes which were heated at 210–220° for 16 hr. The tubes were washed out with *cyclo*hexane (15 ml.) and the combined solutions were distilled in steam. A portion (0·1 ml.) of the *cyclo*hexane layer, after dilution to 40 ml. with ethanol, showed maximum absorption at 222 mμ., optical density 0·425. To the *cyclo*hexane layer semicarbazide hydrochloride (50 mg.) and $NaOAc,3H_2O$ (50 mg.) dissolved in a little water were added, followed by methanol (10 ml.). The mixture was boiled vigorously for 5·5 hr., cooled and diluted with water and concentrated at low pressure. A crystalline solid separated from the aqueous layer. This was collected, washed with water and dried (35 mg.; m.p. 85–105°). Crystallization from benzene–light petroleum afforded plates, m.p. 120–122° (Kofler block). After one recrystallization from aqueous methanol and a final, slow crystallization from 50% (v/v) aqueous ethanol, large diamond-shaped plates were obtained, m.p. 133°, remelting after cooling at 124°; $[\alpha]_D^{20}$ −24·5° (*c* = 1 in dioxan). (Found: C, 64·0; H, 10·6; N, 18·8. $C_{19}H_{23}ON_3$ requires C, 64·0; H, 10·2; N, 18·7%.)

Synthesis of (−)-4:8-dimethylnon-2-en-1-al semicarbazone. Magnesium (2·4 g.) was stirred vigorously with mercury (240 g.) until amalgamation was complete. (+)-Citronellal (15·4 g.; $[\alpha]_D^{20}$ +8°) and ethyl dichloroacetate (15·7 g.) were mixed in dry ether (20 ml.) and about half of this solution was added to the stirred amalgam which had been covered with dry ether (80 ml.). A vigorous reaction soon set in and external cooling was needed to supplement an efficient reflux condenser while the remaining solution was added as rapidly as possible. When the reaction subsided the mixture was stirred for 2 hr. Ice and 2 N-H_2SO_4 were added. The ethereal layer was washed with water and with M-$NaHCO_3$, dried and evaporated. Fractional distillation of the residue gave *ethyl 2-chloro-3-hydroxy-5:9-dimethyldec-8-en-1-oate* (11·4 g.), b.p. 94–97°/0·08 mm., n_D^{20} 1·4731 (Found: C, 61·1; H, 9·3; Cl, 12·5. $C_{14}H_{25}O_3Cl$ requires C, 60·8; H, 9·05; Cl, 12·8%.) If the reaction was conducted more slowly, the yield was lower, perhaps because self-condensation of the citronellal became more important.

The ester (11·4 g.) was treated at room temperature with a slight excess of sodium ethoxide in dry ethanol. Sodium chloride separated at once. After a few minutes, sufficient acetic acid to neutralize the mixture was added. Ether and water were added and the ether-soluble product was distilled. Ethyl 2:3-epoxy-5:9-dimethyldec-8-en-1-oate (7 g.), somewhat impure, was collected at 89–91°/0·06 mm. (Found: C, 69·1; H, 10·3. $C_{14}H_{24}O_3$ requires C, 70·0; H, 10·0%.)

The glycidic ester (7 g.) was saponified by heating for 1 hr. with ethanol (31·5 ml.) and N-NaOH (31·5 ml.). The ethanol was removed at low pressure, a trace of neutral material was extracted with ether and the cold aqueous solution was carefully acidified to Congo red with 2 N-H_2SO_4 in the presence of ether. The crude glycidic acid remained as a thick oil (5·7 g.) on evaporation of the ether. This was hydrogenated in ethyl acetate (96 ml.) over PtO_2 (240 mg.). In less than 1 hr., slightly more than one equivalent of hydrogen was absorbed and reaction then ceased. On removal of catalyst and solvent, crude 2:3-epoxy-5:9-dimethyldecanoic acid (5·7 g.) remained.

This product was dissolved in benzene (75 ml.) and dry HBr was added until an excess was present, which was then removed by thorough washing with water. Evaporation of the benzene *in vacuo* then left crude 3-bromo-2-hydroxy-5:9-dimethyldecanoic acid (7·16 g.). This acid was dissolved in a little benzene and a little of this solution was added to a hot solution of lead tetraacetate (10·95 g.) in benzene (100 ml.). When the initially colloidal separation of lead diacetate had crystallized, the remaining acid was added and the mixture was boiled for a few minutes on the water bath. Liberation of CO_2 was demonstrated at this stage by trapping the evolved gas in baryta. The nearly colourless solution was washed with water and with N-HCl. A precipitate of $PbCl_2$ was removed and the benzene solution after further washing with water was extracted with 0·5 M-$NaHCO_3$. The alkaline extracts were extracted with ether; the ether extract was washed with water and with saturated aq. NaCl and added to the benzene solution, which had also been washed with NaCl. On removal of solvent *in vacuo* a yellow oil (4·05 g.), containing 2-bromo-4:8-dimethylnonan-1-al, remained. The alkaline extracts afforded 2·53 g. of acidic material.

To the crude bromo-aldehyde (4 g.) were added dry methanol (12 ml.) and ethyl orthoformate (3 ml.), followed by dry methanol saturated with HCl (0·25 ml.). Next day the solution was treated with sodium methoxide until neutral. Potassium hydroxide (0·7 g.) was then added and, after 1·5 hr., ether and water. The ether-soluble material (2·9 g.) was distilled to give 1·9 g., b.p. 83–85°/0·5 mm. (Found: C, 55·2; H, 9·1; Br, 22·9. $C_{13}H_{27}O_2Br$ requires C, 53·2; H, 9·1; Br, 27·1%.) If all the bromine in this product is attributable to 2-bromo-4:8-dimethylnonan-1-al dimethyl acetal the purity is 85%. This product (1·8 g.) was refluxed in methanol (9 ml.) containing KOH (0·9 g.) for 40 hr. On

distillation of the neutral ether-soluble product a main fraction (0·65 g.) was obtained, b.p. about 100°/15 mm. This showed no absorption at 223 mμ. in aq. ethanol, but a maximum at this wavelength appeared rapidly on addition of a trace of HCl. Acetic acid (1·8 ml.) was added to the crude acetal (0·65 g.) in ethanol (24 ml.) and water (24 ml.); after 5 min. a solution of semicarbazide hydrochloride and $NaOAc,3H_2O$ (400 mg. each) in a little water was added. Crystallization set in after a few minutes; the product was collected next day and recrystallized from 50 % aq. ethanol to give a first crop of 119 mg. Slow recrystallization from the same solvent produced large plates, m.p. 133–135° (Kofler), $[\alpha]_D^{20}$ − 22° (*c* = 1 in dioxan), of (−)-4:8-*dimethylnon*-2-*en*-1-*al semicarbazone*. (Found: C, 64·2; H, 9·9; N, 18·5. $C_{12}H_{23}ON_3$ requires C, 64·0; H, 10·2; N, 18·7 %.) The evidence for identity of this specimen with the semicarbazone from cholesterol has already been given (Cornforth *et al.* 1954). This semicarbazone absorbed maximally at 265 mμ. in ethanol. On being kept overnight with a solution of pyruvic acid in 85 % acetic acid, the aldehyde was regenerated as a pleasant-smelling oil showing intense absorption at 223 mμ.

At this stage of the work, a report from Zürich appeared (Riniker, Arigoni & Jeger, 1954) in which the preparation of the dihydro derivative of the aldehyde (IX) from cholesterol and from citronellal was described. Since the Swiss workers gave a procedure for ozonolysis of cholest-14-en-3β-yl benzoate and subsequent pyrolysis which seemed more convenient than our own, we adopted their method. By pyrolysis of the keto aldehyde (VIII; R = Bz) a distillate containing the aldehyde (IX) was obtained, and from the residue after saponification, chromatography and acetylation the tricyclic acetoxyketone (XVIII) was separated. Since this ketone has been obtained by unambiguous total synthesis (Cardwell, Cornforth, Duff, Holtermann & Robinson, 1951; Billeter & Miescher, 1950) both fragments of the pyrolysis of (VIII) are securely identified. A crystalline by-product which was present in the non-volatile residue before saponification appeared to be the lactone (XIX); this may have been formed from the keto aldehyde (VIII) by internal oxidation–reduction.

We now desired the unsaturated acid corresponding to (IX), in the expectation that its potassium salt (XX) would undergo αβ-fission on heating with potassium hydroxide, a reaction often put to good use in degradation (Hunter & Popjak, 1951; Cornforth *et al.* 1953*a*; Cornforth & Popjak, 1954). Experiments with synthetic aldehyde (IX) had shown this to resist oxidation by silver oxide, and since it was desirable to avoid more vigorous and less specific oxidizing agents we had recourse to a little-known method peculiarly suited to our purpose. The smooth cleavage of an aldoxime by hot aqueous alkali to give ammonia and the salt of a carboxylic acid:

$$R.CH{:}N.OH + KOH \rightarrow R.CO_2K + NH_3$$

seems first to have been applied by Zanetti (1891) to the succinaldoximes obtainable from pyrroles. The method appears to be rather general: we find, for example, that acetaldoxime is rapidly converted by boiling 30 % potassium hydroxide solution into ammonia and potassium acetate. When the aldehyde (IX) was treated with hydroxylamine and the crude oxime was heated with potassium hydroxide, ammonia was evolved at 100–150°. The temperature was then raised to 350° for a few minutes, when the expected fission of the αβ-unsaturated potassium salt of (XX) took place, the steam-volatile acidic product being shown by gas–liquid chromatography (James & Martin, 1952) to consist entirely of acetic acid and a branched-chain C_9 acid. The process was now repeated with radioactive material; the two acids (XXI) and (XXII) were separated by partition chromatography and shown to occur in nearly equimolecular amount and in about 70 % yield based on the alkali consumed during oximation. These two acids were reserved for isolation of carbon atoms 15, 16 and 17 of cholesterol (see below). Their structure and origin follow unambiguously from the mode of formation, the composition of the silver salt of (XXI), and the gas–liquid chromatographic analysis.

The radioactive acetoxyketones (XVIII; R = Ac) were diluted with an equal amount of non-radioactive product (prepared from the 5:6-dehydro analogue derived from oxidation of cholesteryl acetate dibromide and generously supplied by Dr K. Miescher of Ciba Laboratories, Basle, Switzerland) and degraded further by a procedure based on that of Billeter & Miescher (1950). Bromination in benzene solution, followed by dehydrobromination with pyridine, gave a product consisting largely of the unsaturated ketone (XXIII). This always contained a small proportion of the saturated ketone (XVIII; R = Ac) even when the pure bromide (XXIV) was isolated as an intermediate, some reversal of the bromination presumably being effected by pyridine hydrobromide; complete purification of the ketone (XXIII) would have been expensive but was fortunately unnecessary. Ozonization in ethyl chloride at − 30°, followed by steam-distillation of the product, gave a distillate containing a 50–60 % yield of acetic acid and a trace of formic acid; the two acids were identified by gas–liquid chromatography. The acetic acid was purified by partition chromatography and was reserved for identification of carbon atoms 18 and 13 of cholesterol. Its origin from these two carbons cannot be doubted: the acetoxyl group in (XXIII) should not be appreciably hydrolysed under these conditions, nor could acetic acid thus formed have the radioactivity later detected.

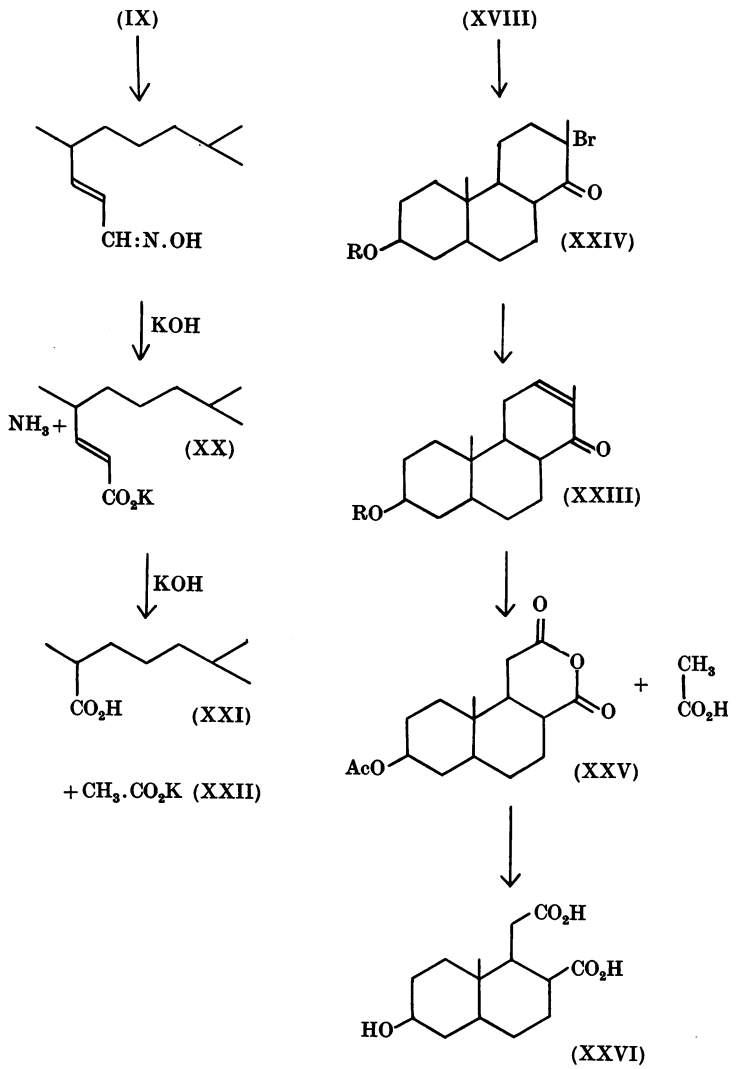
The product non-volatile in steam was further oxidized by hydrogen peroxide; treatment of the

acidic fraction with acetic anhydride then gave the anhydride (XXV) described by Billeter & Miescher (1950), who oxidized the unsaturated ketone (XXIII) with chromic acid in acetic acid. This anhydride on alkaline hydrolysis afforded the hydroxydicarboxylic acid (XXVI), which served as the source of carbons 12 and 14 of cholesterol (see below). Its structure follows from its mode of formation, its composition and the composition of the acetyl derivative and acetylated anhydride previously obtained by Billeter & Miescher.

Ozonolysis of radioactive cholest-14-en-3β-yl benzoates. A solution of the carboxyl-labelled benzoate (6·895 g.) in ethyl chloride (100 ml.) was ozonized in two portions at −70° until excess of ozone was present. The solvent was removed and the residue was recrystallized from methylene chloride by addition of methanol to give the ozonide (6·92 g.), m.p. 128–130°. One-half (3·46 g.) of this product was stirred for 2 hr. on a steam bath with acetic acid (170 ml.) and Zn powder (11·5 g.). The cooled filtered solution was evaporated at low pressure; the residue was dissolved in ether and water. The ethereal layer, after washing with dilute NaOH and aq. NaCl, was dried and evaporated. The crude keto aldehyde (VIII) was used directly for the next stage.

Ozonolysis of methyl-labelled cholest-14-en-3β-yl benzoate (6·49 g.) in similar fashion gave the ozonide (6·635 g.), m.p. 127–130°. One-half (3·3 g.) of this was reduced with Zn and acetic acid as described above.

Pyrolysis of radioactive 3β-benzoyloxy-14-oxo-14:15-seco-cholestan-15-al. The carboxyl-labelled keto aldehyde, obtained as above from 3·46 g. of ozonide, was heated at 15 mm. pressure (capillary leak fed with N_2) to 200°; the temperature was raised during 2 hr. to 250° and maintained



there for 3 hr. The volatile product, trapped in a receiver at −30°, was taken up in a little ether and washed with a few drops of 0·5 M-$NaHCO_3$ to remove benzoic acid. The ether was removed at low pressure and the residue was distilled, b.p. about 110°/15 mm., to give the aldehyde (IX) as a colourless pleasant-smelling oil (251 mg.).

Similar treatment of the methyl-labelled keto aldehyde (from 3·3 g. of ozonide) gave 181 mg. of redistilled aldehyde (IX).

Degradation of radioactive 4:8-*dimethylnon*-2-*en*-1-*al to give acetic acid and* 2:6-*dimethylheptanoic acid.* To the carboxyl-labelled aldehyde (251 mg.) was added hydroxylamine hydrochloride (100 mg.) with a few drops of water and of propan-2-ol and a trace of methyl orange. N-Sodium hydroxide solution was then added slowly as oximation proceeded until the red colour of the indicator failed to return: 1·35 ml. was required (90% of the theoretical). Propan-2-ol was added from time to time to dissolve most of the oil which separated (ethanol instead of propan-2-ol was found in pilot experiments to produce unwanted acetic acid by oxidation during the subsequent heating). The ether-soluble product, after washing with water, was treated with KOH pellets (2·5 g.), a solid yellow potassium salt being formed. The temperature was slowly raised to 350° and kept there for 10 min. Ammonia was evolved from 110–200°. The cooled melt was dissolved in water, acidified with H_2SO_4 and distilled in steam. The distillate was neutralized with NaOH (phenol red) and evaporated at low pressure. The sodium salts were decomposed by addition of 15 N-H_2SO_4 (0·2 ml.); this mixture on a pad of asbestos was placed at the top of a chromatographic column in which 0·5 N-H_2SO_4 supported by Hyflo Super Cel was the stationary phase (cf. Cornforth, Hunter & Popjak, 1953*b*). The column was developed first with $CHCl_3$, successive 5 ml. portions of effluent being titrated with 0·19 N ethanolic LiOH (bromothymol blue). The 2:6-dimethylheptanoic acid ran fast through the column and required 5·17 ml. (0·982 m-mole) for neutralization. Thereafter, $CHCl_3$ containing 10% (v/v) of BuOH was used to elute the acetic acid, which required 6·24 ml. (1·185 m-moles). The lithium salts were recovered by evaporation; that of 2:6-dimethylheptanoic acid was redissolved in water and the silver salt was precipitated by gradual addition of $AgNO_3$, the first portion of precipitate being rejected. The silver salt was dried *in vacuo* over P_2O_5. (Found: for a non-radioactive specimen, C, 40·55; H, 6·9; residue 42·1. $C_9H_{17}O_2Ag$ requires C, 40·8; H, 6·4; Ag, 40·8%.)

Methyl-labelled dimethylnonenal (181 mg.) was put through the same process. During oximation, 0·93 ml. of N-NaOH was consumed (86%). The steam-volatile acids obtained after fusion of the oxime were resolved into dimethylheptanoic acid (0·606 m-mole) and acetic acid (0·725 m-mole).

Radioactive 2:13-*dimethyl*-trans-anti-trans-*perhydrophenanthren*-1-*on*-7β-*yl acetates.* The non-volatile product of pyrolysis from carboxyl-labelled keto aldehyde (VIII), dissolved in a little benzene, was refluxed with 0·5 N methanolic KOH (23 ml.) for 1 hr. The neutral product, recovered in the normal manner, was dissolved in benzene and put on a column of alumina. The column was washed with benzene and then with benzene–ether (9:1). After elution of some gummy material, partly crystalline fractions were obtained totalling 1·35 g. These were combined and acetylated by leaving for 24 hr. with acetic anhydride (5 ml.) and pyridine (2·5 ml.). The neutral product was separated in the usual way and recrystallized from light petroleum (b.p. 40–60°) and then from methanol containing a few drops of water. The acetoxyketone (XVIII, R = Ac) (339·4 mg.) then had m.p. 144–145°.

In a similar fashion the pyrolysate from methyl-labelled keto aldehyde afforded acetoxyketone (XVIII; R = Ac) (297·0 mg.) m.p. 144–145°.

Each of these specimens was mixed with an equal weight of non-radioactive acetoxyketone, m.p. 144–145°, prepared according to Billeter & Miescher (1950).

3β-*Benzoyloxy*-14-*hydroxy*-14:15-seco*cholestan*-15-*oic acid lactone.* After pyrolysis of a portion (2·92 g.) of non-radioactive keto aldehyde (VIII) the non-volatile product in benzene was chromatographed directly on alumina (previously neutralized by washing with methyl formate). Benzene eluted the benzoate of the tricyclic ketone; thereafter, benzene–ether (3:1) eluted a product which after two recrystallizations from ethanol formed colourless needles (0·11 g.), m.p. 183·5°. (Found: C, 78·2; H, 9·7. $C_{34}H_{50}O_4$ requires C, 78·2; H, 9·6%.) The infrared spectrum (in compressed KBr) showed carbonyl absorption at 1713 and 1745 $cm.^{-1}$. This confirmed that the *lactone* (XIX) had been isolated.

Radioactive 2:13-*dimethyl*-trans-anti-trans-1:4:5:6:7:8:-9:10:11:12:13:14-*dodecahydrophenanthr*-1-*on*-7β-*yl acetates.* Carboxyl-labelled acetoxyketone (XVIII; R = Ac; 0·6788 g.) was dissolved in thiophen-free benzene (25 ml.), cooled in ice and treated gradually with a solution of bromine in benzene, each portion being allowed to react before adding the next (5·65 ml. of 0·845 N). The solution was washed with 0·5 M-$NaHCO_3$ and the benzene was evaporated at low pressure. The crystalline residue was boiled with pyridine (4·5 ml.) for 10 min. and the solution was poured into 2 N-HCl (30 ml.). The neutral product was isolated and crystallized at −5° from hexane to give the unsaturated ketone (XXIII) (0·54 g.), m.p. 101–103·5°.

The methyl-labelled analogue (0·525 g.; m.p. 102–105°) was prepared similarly from the saturated ketone (0·5940 g.).

Ozonolysis of radioactive unsaturated ketone (XXIII). Carboxyl-labelled unsaturated ketone (0·500 g.) was dissolved in ethyl chloride (15 ml., previously treated with ozone and washed with 0·5 M-$NaHCO_3$), cooled to −30° and ozonized for 3 hr. Ozone was still present in solution after keeping at −70° overnight. The ozone was displaced by a current of air and the solvent was evaporated. The residue after warming with a little water was distilled in steam. The distillate after neutralization with NaOH was evaporated; vapour-phase chromatography of the acidified residue indicated the presence of acetic acid and a trace of formic acid. Partition chromatography by the technique already described (5% butanol in $CHCl_3$ was used) separated the acetic acid (1·056 m-moles), which was reserved as the lithium salt.

Ozonolysis of the methyl-labelled analogue (0·4118 g.) in the same manner yielded acetic acid (0·725 m-mole).

Radioactive 1-*carboxymethyldecahydro*-6-*hydroxy*-9-*methyl*-2-*naphthoic acids.* The fraction non-volatile in steam, from ozonization of carboxyl-labelled unsaturated ketone (500 mg.), was extracted with ether and $CHCl_3$; it weighed 450 mg. This was dissolved in acetic acid (3 ml.) and the solution was added slowly to a mixture of 30% H_2O_2 (0·3 ml.), water (0·6 ml.) and conc. H_2SO_4 (0·015 ml.); the whole was then refluxed for 1 hr., diluted with water

(16 ml.) and extracted thrice with ether. The ether layer was washed with water and the solvents were evaporated, finally *in vacuo* over KOH. The residue was dissolved in ether and extracted with 3 × 5 ml. of 2N-NaOH. On evaporation of the ether, a residue (19·7 mg.) remained which consisted chiefly of the saturated ketone (XVIII; R = Ac). The alkaline extract was acidified to Congo red with 2N-HCl and extracted continuously with ether. The extracted product (208 mg.) was dissolved in dry pyridine (1·7 ml.) and acetic anhydride (0·85 ml.). Next day the mixture was evaporated at low pressure, ether and water were added, and the ether, after washing with 0·5M-$NaHCO_3$ and water, was evaporated. The residue (185 mg.) crystallized in contact with CCl_4. It was left for some months at this stage, during which time a little hydrolysis occurred; this was corrected by warming with acetic anhydride (1 ml.) at 100° for 1 hr. After concentration at low pressure the product was recrystallized from CCl_4 to give 6-acetoxy-1-carboxymethyldecahydro-9-methyl-2-naphthoic acid anhydride (83 mg.) in prisms, m.p. 193°. This material was boiled for 2 hr. with 0·5N-NaOH (2·00 ml.); N-HCl (1 ml.) was then added and the solution was quickly concentrated at low pressure. The *acid* (XXVI) crystallized in small colourless prisms (70 mg.). [Found for a similarly prepared non-radioactive specimen: m.p. 234–236° (decomp.); $[\alpha]_D^{23}$ + 4° [*c* = 0·5 in $CHCl_3$–ethanol]. Found: C, 62·1; H, 8·4. $C_{14}H_{22}O_5$ requires C, 62·2; H, 8·2%.]

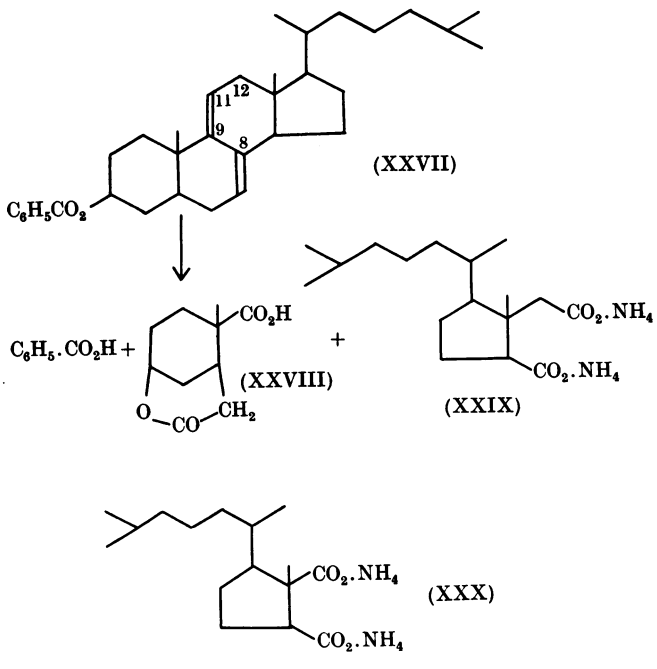
The methyl-labelled analogue was prepared in analogous fashion; the yield of anhydride was 55 mg. and of dicarboxylic acid 46 mg.

The second degradation

At this stage we had some hope of continuing the degradation of cholesterol by further treatment of the acid (XXVI), for example by interaction of an ester with phenylmagnesium bromide. Pilot experiments with non-radioactive material showed, however, that interaction with both ester groups was difficult to attain, and finally we turned to a different plan. The execution of this required more radioactive 7-dehydrocholesterol; in order to save the labour of repeating this preparation we recrystallized non-radioactive 7-dehydrocholesterol in the mother liquors from which radioactive 7-dehydrocholesterol had separated. This expedient afforded material of low but sufficient radioactivity. Benzoylation, followed by hydrogenation in benzene over Raney nickel, then gave cholest-7-en-3β-yl benzoate (III; R = Bz).

The preparation of cholesta-7:9(11)-dien-3β-yl benzoate (XXVII) by oxidation of a 7-ene with mercuric acetate has twice been reported (Fieser, Herz & Huang, 1951; Heusser, Heusler, Eichenberger, Honegger & Jeger, 1952). Anderson, Stevenson & Spring (1952) have shown in the ergostane series that dehydrogenation by bromine (followed by sodium iodide) gives purer products than does the mercuric acetate method. Our adaptation of their procedure to cholest-7-en-3β-yl benzoate gave, in fact, a diene (XXVII) showing much higher absorption in the ultraviolet than was reported by the Swiss workers (and confirmed by us) for the mercuric acetate product, which must be regarded as impure.

Ozonolysis of the diene benzoate (XXVII) in ethyl chloride at −45° was followed by treatment of the product with hydrogen peroxide in acetic acid containing a little sulphuric acid. Three main products were obtained: benzoic acid, a water-



soluble acid slowly extractable by ether, and a light-petroleum-soluble acid precipitable as an amorphous sodium salt.

The water-soluble acid appeared to be a mixture of the lactone–acid (XXVIII) and the corresponding dicarboxylic acid, for when it was melted at 180° water was expelled and the high-melting lactone–acid crystallized and could be purified by sublimation. The structure (XXVIII) for the lactone–acid is established by its composition and properties; in particular, an isomeride in which C-9 (of cholesterol) is lactonized and C-7 free is stereochemically impossible without an (unlikely) inversion at C-3 or C-10.

The acid from the insoluble sodium salt was converted into an anhydride, which was distilled; the regenerated acid, itself an oil, was purified by crystallization of its ammonium salt, for which analysis indicated the formula (XXIX). This attribution is supported by the following arguments: (1) a dicarboxylic acid of this structure is the expected product of ozonolysis under the mild conditions employed, and is complementary to the lactone–acid already isolated; (2) the only other acid of at all similar composition which can be postulated to arise from this ozonolysis is the nor-dicarboxylic acid (XXX), and the analytical data do not agree with this interpretation.

A rather similar but less direct fission of the ergosterol ring system has been described (Heusser, Beriger, Anliker, Jeger & Ruzicka, 1953), nine stages from the 7:9(11)-diene being required to disrupt the molecule.

Radioactive cholesta-5:7-dien-3β-yl benzoates. The mother liquor from which carboxyl-labelled cholesta-5:7-dien-3β-ol had separated (see above) was evaporated. To the residue (1·37 g.) non-radioactive cholesta-5:7-dien-3β-ol (3·10 g.) was added in ether and the mixture was evaporated. The residue was dissolved in pyridine (21 ml.) and benzoyl chloride (3 ml.) was added. After 60 hr., acetone (6·7 ml.) and water (3·4 ml.) were added; ½ hr. later the mixture was poured into water and extracted with $CHCl_3$. The neutral product, recovered in the usual manner, was boiled with acetone, cooled and collected, to give 3·189 g., m.p. 139–140°, ϵ_{282} 12800.

The residue (1·54 g.) from the mother liquor of methyl-labelled cholesta-5:7-dien-3β-ol was diluted with non-radioactive dienol (3·11 g.) and the product was benzoylated as above to give 3·225 g., m.p. 139–140°, ϵ_{282} 12950.

Radioactive cholest-7-en-3β-yl benzoates. The carboxyl-labelled diene benzoate (3·18 g.) in benzene (200 ml.) was hydrogenated over Raney nickel for 16 hr. in the manner described above for the corresponding alcohol. The product was recrystallized from acetone to give cholest-7-en-3β-yl benzoate (2·516 g.), m.p. 155° (clear at 174°).

The methyl-labelled analogue (2·60 g.) was obtained similarly; m.p. 156° (clear at 176°).

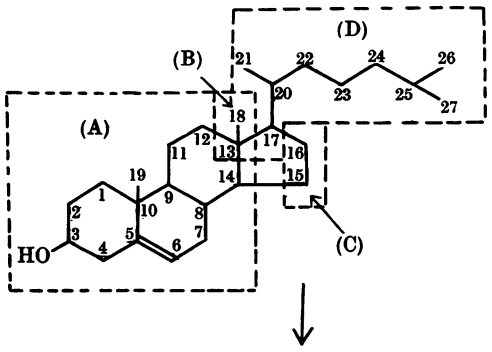
Radioactive cholesta-7:9(11)-dien-3β-yl benzoates. To carboxyl-labelled cholest-7-en-3β-yl benzoate (2·50 g.) in dry ether (250 ml.) at 0° was rapidly added dry bromine (0·825 ml.) in acetic acid (8·25 ml.). The mixture was cooled quickly to −60°, then warmed gradually to 0° during 2 hr. and concentrated somewhat at low pressure. The solid bromo compound (2·2 g.) was collected, dissolved in warm benzene (150 ml.) and treated with NaI (8 g.) in ethanol (150 ml.). Iodine was liberated immediately. After 20 hr. water (150 ml.) was added. The aqueous layer was washed with benzene (100 ml.) and the combined benzene solutions were shaken with 0·25 N-NaOH (2 × 60 ml.), washed with water and evaporated at low pressure. The residue, after two crystallizations from chloroform–methanol, gave cholesta-7:9(11)-dien-3β-yl benzoate (1·458 g.) m.p. 135–136°, showing maximum absorption at 234 mμ. (ε 31500).

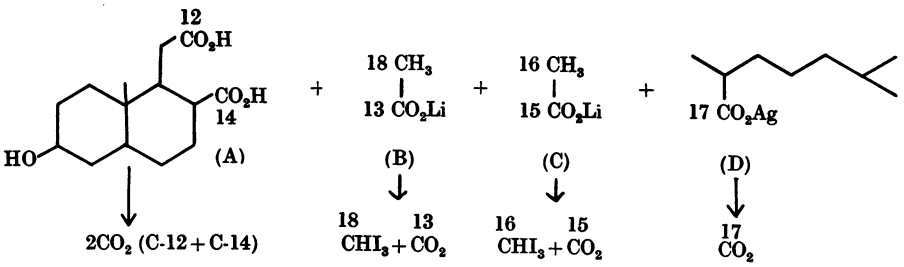
By the same procedures, methyl-labelled cholest-7-en-3β-yl benzoate (2·6 g.) gave cholesta-7:9-dien-3β-yl benzoate (1·406 g.), m.p. 136–136·5°, ϵ_{234} 30 000. A non-radioactive specimen prepared similarly had $[\alpha]_D^{22.5}$ +48°. (Found: C, 83·8; H, 9·9. Calc. for $C_{34}H_{46}O_2$: C, 83·6; H, 9·9%.) Heusser *et al.* (1952) gave m.p. 132–133·5°, $[\alpha]_D^{19}$ +52°, and maximum absorption at 228 mμ., ε 19 950, for material prepared by means of mercuric acetate. Fieser *et al.* (1951) gave m.p. 134°, $[\alpha]_D$ +32° (dioxan).

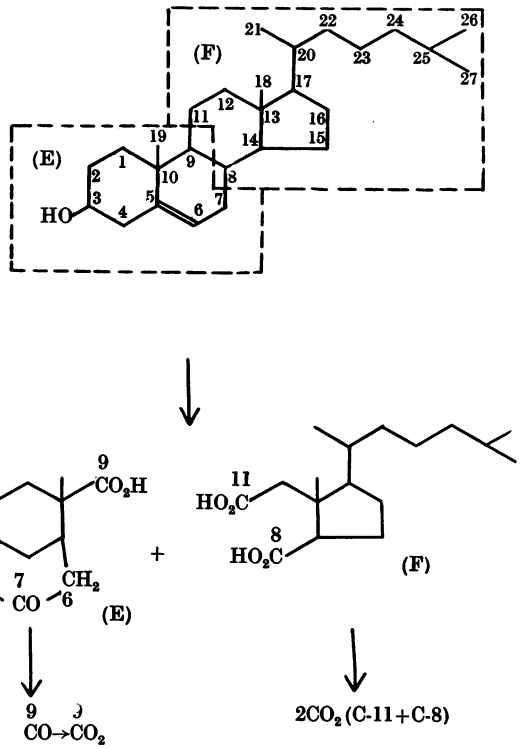
Ozonolysis of radioactive cholesta-7:9(11)-dien-3β-yl benzoates. Carboxyl-labelled diene benzoate (1·4 g.) was dissolved in ethyl chloride (77 ml.) and ozonized at −45° until the solution remained blue. Ozone was displaced by a stream of air and the solvent was removed. The residue was dissolved in acetic acid (11·2 ml.) and added gradually to 30% H_2O_2 (1·12 ml.) in water (2·24 ml.) and concentrated H_2SO_4 (0·07 ml.). The mixture was refluxed for 2·5 hr. and cooled. After addition of $NaOAc,3H_2O$ (170 mg.) the solvents were removed at low pressure, finally at 0·5 mm. To the residue were added ether and 2 N-NaOH (100 ml.). A sodium salt separated in the ether and was dissolved by washing twice with water. The combined solutions were acidified to Congo red with HCl and extracted twice with ether (total about 50 ml.). The aqueous layer (*A*) and the ethereal layer (*B*) were separated.

Layer *A* was extracted continuously with ether for 48 hr. The extracted product was dissolved in acetone, concentrated to crystallizing point and treated with about 4 vol. of benzene. The product, m.p. 150–160°, was collected, washed with acetone–benzene (1:3) and heated to 180–190°/100 mm. Effervescence ceased after about 10 min. and the melt solidified. A cold-finger condenser was inserted; the pressure was lowered to 15 mm. and the temperature raised to 250°. The product sublimed rapidly; it was dissolved in the minimum (30 ml.) of boiling xylene. After cooling, the large, blade-like crystals were collected and washed with benzene to give 183 mg., m.p. 248–250°, of 6-*methyl*-2-*oxa*bicyclo-[3:3:1]-*nonan*-3-*one*-6-*carboxylic acid* (XXVIII). A non-radioactive specimen had m.p. 248–250°, $[\alpha]_D^{25}$ −12·5°. (Found: C, 60·9; H, 6·9%; equiv. 198. $C_{10}H_{14}O_4$ requires C, 60·6; H, 7·1%; equiv. for 1 CO_2H, 198). The infrared spectrum in compressed KCl showed maxima at 1720 cm.$^{-1}$ (lactone CO) and 1680 cm.$^{-1}$ (carboxyl CO).

Layer *B* was evaporated and benzoic acid was sublimed from the residue at 100°/15 mm. To the remainder, 2·5 N-NaOH (10 ml.) was added and the mixture warmed for 1 hr. on a steam bath. The sodium salt (which had separated almost at once) was collected from the hot solution, washed with warm 2·5 N-NaOH and dissolved in water. The free acid was liberated and extracted with light petroleum (b.p. 40–60°); evaporation of the solvent left an oil (263 mg.) which







was boiled for 45 min. with acetic anhydride (4 ml.). The product was recovered by evaporation and distilled at 150–200° (heating block) and 0·04 mm. The distillate (188 mg.) was hydrolysed by warming for 4 hr. on a steam bath with 0·1 N-NaOH (13·2) and the acid (203 mg.) was recovered by means of light petroleum. It was dissolved in a minimum of dilute aq. NH_3; concentrated aq. NH_3 (sp.gr. 0·880) was then added, when the *diammonium salt of* 1-(1:5-*dimethylhexyl*)-2-*methyl*cyclo*pentane*-2-*carboxymethyl*-3-*carboxylate* (XXIX) crystallized in fine colourless needles (144 mg.), which were collected by centrifuging at 0° and dried over KOH in an atmosphere containing NH_3. A non-radioactive specimen had $[\alpha]_D^{23\cdot5} + 50°$ ($c = 1$ in water). (Found: C, 61·8; H, 10·8; N, 8·0. $C_{17}H_{36}O_4N_2$ requires C, 61·4; H, 10·8; N, 8·4%.) An ammonium salt of the nor-dicarboxylic acid (XXX), $C_{16}H_{34}O_4N_2$, requires C, 60·4; H, 10·7; N, 8·8%. The salt melted and decomposed at about 100°, and was unstable *in vacuo*, losing NH_3 and reverting to a gum.

Methyl-labelled cholesta-7:9(11)-dien-3β-yl benzoate (1·37 g.) was ozonized and worked up in essentially the same manner, but less profitably, 120 mg. of lactone (XXVII), m.p. 248–250°, and 88 mg. of ammonium salt (XXIX) being obtained.

Further degradation to one-carbon fragments

Having now recorded the cleavage of cholesterol into six fragments of convenient structure, we shall describe the separation from these of the carbon atoms whose biosynthetic origin was to be determined. The progress from cholesterol to the six

fragments lettered (A) to (F), and thence to one-carbon compounds representing various positions in the molecule of cholesterol, is shown diagrammatically on p. 104.

The two specimens of lithium acetate (B and C) were converted into barium carbonate (representing the carboxyl groups) and iodoform (representing the methyl groups) by a well-established procedure (cf. Popjak, 1955). The silver salt (D) of the C_9 acid (XXIII) on treatment with bromine readily gave up carbon dioxide, which was collected as barium carbonate. Thus carbon atoms 13, 15, 16, 17 and 18 of cholesterol had been isolated.

The lactone–acid (E), on warming in concentrated sulphuric acid at 95°, liberated carbon monoxide, which was oxidized with iodine pentoxide to carbon dioxide and finally converted into barium carbonate. The yield was 60–70 %. Loss of carbon monoxide under these conditions is a characteristic of tertiary carboxylic acids, as was shown originally by Bistrzycki (cf. Bistrzycki & Mauron, 1907, and earlier papers there cited); thus it is certain that this carbon monoxide derived ultimately from C-9 of cholesterol and not from C-7, the other (potential) carboxyl group being primary.

More difficulty was caused by the dicarboxylic acids (A and F). Although the carboxyl groups in these acids are non-equivalent and procedures could no doubt be devised to eliminate them separately, lack of material made us decide for complete decarboxylation. The carbon dioxide from each acid would then represent two different carbon atoms in the molecule of cholesterol. Hence the biosynthetic origin of the individual carbon atoms could not be deduced from measurements of radioactivity on this carbon dioxide unless two conditions were satisfied. First, the yield of carbon dioxide must be high enough to make certain that both carboxyl groups contributed to it. Secondly, if the carbon dioxide was inactive (or nearly so) when an acid deriving from one type of cholesterol (e.g., methyl-labelled) was decarboxylated, then decarboxylation of the acid from the other type of cholesterol must give radioactive carbon dioxide having approximately the degree of radioactivity expected for labelled positions. Had the carbon dioxide from both types of cholesterol been radioactive, or had an unexpected level of radioactivity been found, it would then have been necessary to take the carboxyl groups separately.

The Schmidt reaction was tried first, but it was found impossible, despite many variations in technique, to raise the yield of carbon dioxide from either dicarboxylic acid above 50 % of the theoretical for two carboxyl groups. Bromine on the silver salt of dicarboxylic acid (F) gave only a 30 % yield of carbon dioxide. The method finally adopted was that of anodic decarboxylation. Electrolysis of carboxylic acid salts has been known for more than a century to cause decarboxylation; an excellent review is given by Weedon (1952). The process can logically be formulated as the discharge of a carboxylate ion and decomposition of the carboxylate radical to give carbon dioxide and an alkyl radical which then dimerizes, disproportionates or reacts with another molecule.

$$R.CO_2^- - e \rightarrow R.CO_2^{\cdot} \rightarrow R^{\cdot} + CO_2.$$

It is often difficult to obtain a high yield of an individual product arising from the radical R·, but the only known side reaction which diminishes the yield of carbon dioxide is that which leads to an ester,

$$2R.CO_2^- - 2e \rightarrow R.CO_2R + CO_2,$$

and this is usually of little import.

For the present purpose, only the carbon dioxide was required. The acid (A) was electrolysed in aqueous pyridine, the acid (F) in aqueous pyridine containing a little triethylamine to improve conductivity. The yield of carbon dioxide isolated as barium carbonate was 70 % for A, and 75–80 % for F, of the theoretical value for two carboxyl groups.

Degradation of lithium acetate specimens [*(B) = carbons* 18 + 13] *and* [*(C) = carbons* 16 + 15]. These were carried out by pyrolysis *in vacuo* (10^{-4} mm. Hg) to give finally $BaCO_3$ from the CO_2H and CHI_3 from the methyl carbons. From lithium acetate (B) of carboxyl-labelled cholesterol 75 mg. of CHI_3 (C-18) and 41 mg. of $BaCO_3$ (C-13), and from methyl-labelled cholesterol 63 mg. of CHI_3 and 36·6 mg. of $BaCO_3$, were obtained. Lithium acetate (C) from carboxyl-labelled cholesterol gave 106 mg. of CHI_3 (C-16) and 63·6 mg. of $BaCO_3$ (C-15), and from methyl-labelled cholesterol 37 mg. of CHI_3 and 38 mg. of $BaCO_3$ (the centrifuge tube in which this sample was being washed broke and the CO_2 had to be regenerated *in vacuo* and trapped in fresh baryta).

Decarboxylation of silver salts (D). To a fine suspension of the dry carboxyl-labelled silver salt (150 mg.) in dry CCl_4 (2 ml.) was added bromine (0·09 g.) in CCl_4 (0·3 ml.). Carbon dioxide was evolved and, after passing a short air condenser, was swept by CO_2-free N_2 into half-saturated $Ba(OH)_2$. Finally the suspension was boiled to complete the reaction and to drive over the remaining CO_2. The yield of $BaCO_3$ was 61 mg. Methyl-labelled silver salt (120 mg.) gave 44 mg. of $BaCO_3$ by the same procedure.

Decarboxylation of lactone–acids (E). Carboxyl-labelled lactone–acid (72 mg.) was dissolved in concentrated H_2SO_4 (2 ml.) and heated to 95°. The liberated gas was swept by CO_2-free N_2 through soda-lime (to absorb SO_2 and CO_2), then through a tube of I_2O_5 maintained at 120°, and finally through half-saturated $Ba(OH)_2$. Iodine was formed from the I_2O_5 and $BaCO_3$ precipitated in the $Ba(OH)_2$. After 75 min. heating was discontinued; after a further 45 min. the $BaCO_3$ (44 mg.) was collected. Methyl-labelled lactone–acid (66·5 mg.) by the same procedure gave $BaCO_3$ (42·7 mg.).

Anodic decarboxylation of acids (A). The electrolytic cell was a small tube fitted by means of a B14 standard ground-glass joint to a cap carrying gas inlet and outlet tubes and

two platinum wires, sealed through the glass, which projected to the bottom of the cell and were maintained 3 mm. apart by a glass support fused around them. The cell was charged with carboxyl-labelled acid (A) (30·8 mg.) in pyridine (0·1 ml.) and water (0·2 ml.) and a potential of 12 v was applied to the electrodes. The cell was cooled externally by water and the gas evolved was swept by CO_2-free N_2 into half-saturated $Ba(OH)_2$. The initial current was 34 mA; this dropped steadily as carboxylate ions were eliminated, and after 62 min. was 6 mA; it was computed graphically that 52 coulombs had passed. The circuit was broken and the cell was heated in boiling water for ½ hr. The $BaCO_3$ after collection weighed 31·5 mg.

Methyl-labelled acid A (33 mg.) gave $BaCO_3$ (33 mg.) by the same procedure.

Anodic decarboxylation of acids (F). The ammonium salt (45·3 mg.) of carboxyl-labelled acid (F) was decomposed by ether and 0·1 N-HCl. The acid was transferred quantitatively to the electrolytic cell and dissolved in pyridine–water (3:1; 0·3 ml.). Triethylamine (0·025 ml.) was added and a potential of 24 v was applied. The initial current was 48 mA; this diminished during ½ hr. to a steady value of 18 mA. After 40 min. (61 coulombs) the current was stopped and the cell was heated in boiling water for ½ hr.; after cooling, only 7 mA passed on applying 24 v to the electrodes, showing that the triethylamine, unlike pyridine, had retained some CO_2 at room temperature. The $BaCO_3$ after collection weighed 40·1 mg. Methyl-labelled ammonium salt (43·4 mg.) gave $BaCO_3$ (42·3 mg.) in the same way.

Measurements of radioactivity. The samples of $BaCO_3$ and CHI_3 from acetic acids (B) and (C) and of $BaCO_3$ from the silver salt (D) were counted on plastic planchets (Popjak, 1950) with a thin mica-window counter. Carboxyl-labelled and methyl-labelled cholest-14-en-3β-yl benzoates were counted in the same way. Appropriate correction for the greater back-scatter of β-rays from the $BaCO_3$ and CHI_3 samples as compared with the cholestenyl benzoate samples were made. The counts for these samples are given in Table 1 as counts/min. at infinite thickness carbon, these being proportional to the specific activity of the carbon.

The $BaCO_3$ from the lactone–acids E and the dicarboxylic acids (A) and (F) was decomposed in Rittenberg tubes and the CO_2 was counted in a proportional gas counter (Bradley, Holloway & McFarlane, 1954). Carbon dioxide from combustion of acids (A), (E) and (F) was counted in the same apparatus. The counts obtained (Table 2) were calculated, on the basis of the known efficiency of the gas counters used, in terms of number of disintegrations/min./mg. of carbon.

RESULTS AND DISCUSSION

Degradation of ring D and identification of carbons 13, 15, 16, 17 and 18 were completed in 1954, and the results have been briefly reported (Cornforth, 1954; Cornforth, Popjak & Gore, 1956). Table 1 shows that of these five carbon atoms, only one, C-16, originates from the carboxyl carbon of acetate and all the others from the methyl carbon. When [*carboxy*-^{14}C]acetate was the precursor of cholesterol C-13, C-15, C-17 and C-18 contained no detectable radioactivity, as previously reported (Cornforth *et al.* 1953*b*) for C-1, C-3, C-5 and C-10. On the other hand, when [*Me*-^{14}C]acetate was the precursor, two types of labelling were found: one with high radioactivity (C-13, C-15, C-17 and C-18) and another (C-16) with low but significant activity. This duality was also found in the earlier work with ring A and was attributed to the conversion of some [*Me*-^{14}C]acetate into [*carboxy*-^{14}C]acetate by way of the tricarboxylic acid cycle; in the present experiments, however, this effect was less marked than before. As mentioned earlier, the same biosynthesis from [*Me*-^{14}C]acetate which provided us with methyl-labelled cholesterol was also used to prepare methyl-labelled squalene, the degradation of which has been reported (Cornforth & Popjak, 1954). The same two types of labelling were found in this squalene, and the ratio of the radioactivities was close to that now found in cholesterol.

In the second set of degradations, aiming at C-9, C-11, C-8, C-12 and C-14, it has not been possible to relate all the results to a single parent compound, as some of the fragments from which these carbon atoms were obtained have of necessity been diluted to different and unpredictable degrees in the course

Table 1. *Specific activity (counts/min./mg. of* C) *of carbons* 15, 16, 17, 18 *and* 13 *of cholesterol biosynthesized from* [carboxy-^{14}C]*acetate and from* [Me-^{14}C]*acetate*

Precursor of cholesterol ...	Counts/min./mg. of C	
	$CH_3.{}^{14}CO_2H$*	$^{14}CH_3.CO_2H$*
Cholest-14(15)-enol	568	733
C-15	Inactive	1080†
C-16	1336	98
C-17	Inactive	1279
C-18	Inactive	1386
C-13	Inactive	1271

* The two samples of cholesterol were biosynthesized by two different batches of liver slices, and were diluted by inactive material to a different degree. The specific activity of the labelled carbon atoms in the two samples, calculated on the assumption that 12 carbon atoms of cholesterol originate from the carboxyl carbon and 15 from the methyl carbon of acetate, were 1275 counts/min./mg. of C (precursor: $CH_3.{}^{14}CO_2H$) and 1238 counts/min./mg. of C (precursor: $^{14}CH_3.CO_2H$).

† This sample of $BaCO_3$ was recovered from the accident mentoned above (p. 105).

Table 2. *Specific activity (disintegrations/min./mg. of* C) *of* C-9, C-11 + C-8, *and of* C-12 + C-14 *and of the total carbon of the fragments from which they originated*

Precursor of cholesterol ...	Disintegrations/min./mg. of C	
	$CH_3.{}^{14}CO_2H$	$^{14}CH_3.CO_2H$
Lactone acid (fragment E)	5·1	17·8
C-9 (from fragment E)	Inactive	26·8
Dicarboxylic acid (fragment F)	5·7	16·0
C-8 + C-11 (from fragment F)	11·0	1·1
Dicarboxylic acid (fragment A)	330·0	277·0
C-12 + C-14 (from fragment A)	508·0	19·9

of chemical manipulation. Instead, we present in Table 2 specific activities of the total carbon of the various fragments and of the individual carbon atoms derived from these.

The results collected in Table 2 show that C-9 is a methyl carbon and that the other carbons, C-8, C-11, C-12 and C-14, are carboxyl carbons. If we denote a methyl carbon by m and a carboxyl carbon by c, the now complete pattern in cholesterol is as shown (below).

Additional evidence for the origin of the four carbons, C-8, C-11, C-12 and C-14 was desirable, for the method of anodic decarboxylation by which they were separated is new in this context. Although the disappearance of carboxyl groups during electrolysis (as shown by the fall in conductivity) paralleled the evolution of CO_2, and although one can certainly conclude that all or nearly all this CO_2 originated from carboxyl carbons and none from methyl carbons, it was still possible to doubt that all the CO_2 came from the carboxyl groups of the parent acids. Further evidence not dependent upon this assumption was desirable, and was provided by examining the total radioactivity of the fragments (A), (E) and (F).

Fragment (A) is a product of the first degradation, the results of which are given in Table 1. We calculated the average specific activity of the three methyl carbons C-13, C-17 and C-18, and compared this with the specific activity of the carboxyl carbon C-16, obtaining the proportions given in Table 3.

We made the assumption, justified by the present and all previous work on degradation of cholesterol biosynthesized from acetate, that these proportions are substantially true of the other methyl and carboxyl carbons in these two specimens of cholesterol, and hence in the two specimens of fragment (A) which derive from them.

Now if (A) contains eight carboxyl and six methyl carbons, as indicated by the results in Table 2 taken in conjunction with earlier work, it follows that the ratio of the specific activity of the total carbon in carboxyl-labelled (A) to that of methyl-labelled (A) should be

$$\frac{1{\cdot}02\times 8}{14}\Big/\left(\frac{(1{\cdot}00\times 6)+(0{\cdot}075\times 8)}{14}\right)=1{\cdot}24.$$

The ratio found by experiment (Table 2) is 1·19, in satisfactory agreement. If one of the carbons C-8, C-11, C-12 or C-14 were a methyl carbon the calculated ratio would be 0·95; if one of these carbons were not derived from acetate at all, the ratio would be 1·09. Neither of these alternatives agrees so well with experiment.

Similar calculations were applied to fragments (E) and (F). These are fragments of the second degradation, and the dilutions of methyl-labelled and carboxyl-labelled material are different, so that the ratios given in Table 3 are not directly applicable. However, the origin of all carbons in (E) is known: it has six methyl and four carboxyl carbons. If one again sets 1·00 as the specific activity of a methyl carbon in methyl-labelled fragment (E), then the relative activity of a carboxyl carbon in the same specimen should still be 0·075 [since (E) originated from the same specimen of biosynthetic cholesterol as did the fragments of the first degradation], but the relative activity of a carboxyl carbon in carboxyl-labelled (E) must be calculated from the observed values (Table 2) for total carbon in the two specimens of (E), and is found to be

$$\frac{(6\times 1{\cdot}00)+(4\times 0{\cdot}075)}{10}\times\frac{5{\cdot}1}{17{\cdot}8}\times\frac{10}{4}=0{\cdot}45.$$

Thus we have in (E) the proportions of Table 4.

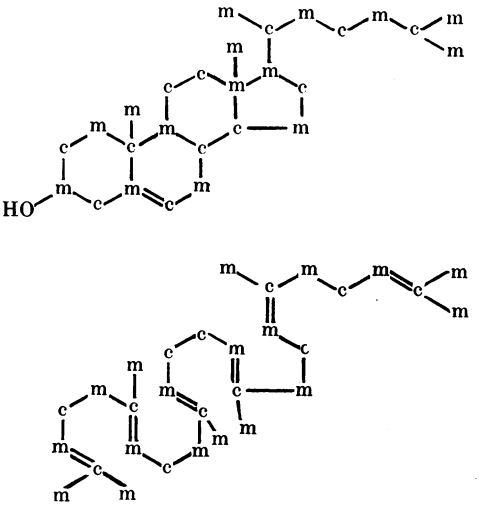


Table 3. *Relative specific activities of methyl and carboxyl carbons in methyl-labelled and carboxyl-labelled cholesterol, calculated from Table* 1

	Methyl-labelled cholesterol	Carboxyl-labelled cholesterol
Individual carboxyl carbon	0·075	1·02
Individual methyl carbon	1·00	0

Table 4. *Relative specific activities of methyl and carboxyl carbons in methyl-labelled and carboxyl-labelled fragment (E), calculated from Table* 2

	Methyl-labelled E	Carboxyl-labelled E
Individual carboxyl carbon	0·075	0·45
Individual methyl carbon	1·00	0

These results are now applicable to fragment (F), a product of the same degradations. If the evidence of electrolysis is valid, this contains nine methyl carbons and eight carboxyl carbons. The ratios of the specific activity of total carbon in methyl-labelled (F) to that in carboxyl-labelled (F) should then be

$$\frac{(9 \times 1{\cdot}00) + (8 \times 0{\cdot}075)}{17} \Big/ \left(\frac{(0{\cdot}45 \times 8)}{17}\right) = 2{\cdot}67.$$

The experimental value (Table 2) is 2·81. If (F) contained ten methyl carbons, the calculated ratio would be 3·34; if it contained nine methyl carbons, seven carboxyl carbons and one carbon not derived from acetic acid, the calculated ratio would be 3·02. Again the agreement is satisfactory if C-8, C-11, C-12 and C-14 are all carboxyl carbons, but is less satisfactory on other hypotheses. Earlier evidence (Little & Bloch, 1950; Würsch, Huang & Bloch, 1952; Cornforth *et al.* 1953*b*) indicating that cholesterol has 15 methyl and 12 carboxyl carbons supports the same conclusion.

The specimens of carbon dioxide obtained by electrolysis of (A) and (F) have 88% and 87% respectively of the specific activities calculated for carboxyl carbon in the parent acids; the carbon dioxide from C-9 has 95% of the specific activity calculated for methyl carbon. These slight but significant deviations from the calculated figures might be attributable to an isotope effect.

The completed pattern of methyl and carboxyl carbons in cholesterol is shown on p. 107 alongside the pattern previously discerned in squalene. The cyclization of squalene to cholesterol postulated by Woodward & Bloch (1953), whereby a methyl carbon migrates to C-13 to give a trimethylsteroid from which three methyl carbons are then eliminated, would lead to an arrangement of methyl and carboxyl carbons in cholesterol identical with that determined by experiment.

SUMMARY

1. Cholesterol biosynthesized by liver slices from [*carboxyl*-^{14}C] acetate and from [*Me*-^{14}C] acetate was chemically degraded by two diverging sequences of reactions.

2. From these two degradations, six fragments were obtained. The structure of one fragment was confirmed by synthesis.

3. From these fragments, one-carbon compounds originating from specified carbon atoms of cholesterol were prepared and their radioactivity was measured.

4. It is concluded that in cholesterol biosynthesized from acetate C-8, C-11, C-12, C-14 and C-16 originate from the carboxyl group of acetate and C-9, C-13, C-15, C-17 and C-18 originate from the methyl group.

5. The origin of all 27 carbon atoms in cholesterol biosynthesized from acetate is now known. The results are shown to harmonize with the current theory that squalene is a precursor of cholesterol in the animal body.

We wish to thank Glaxo Laboratories Ltd. for generous supplies of 7-dehydrocholesterol; Dr K. Miescher of CIBA for a gift of the Köster-Logemann tricyclic ketone; Dr A. T. James for liquid–vapour chromatography of some fatty acid mixtures; Mr G. Savage for the gas counting; and Mr J. Grant for technical assistance.

REFERENCES

Achtermann, T. (1934). *Hoppe-Seyl. Z.* **225**, 141.
Anderson, R. C., Stevenson, R. & Spring, F. S. (1952). *J. chem. Soc.* p. 2901.
Bernstein, S., Binovi, L. J., Dorfman, L., Sax, K. J. & Subbarow, Y. (1949). *J. org. Chem.* **14**, 439.
Billeter, J. R. & Miescher, K. (1950). *Helv. chim. acta*, **33**, 388.
Bistrzycki, A. & Mauron, L. (1907). *Ber. dtsch. chem. Ges.* **40**, 4370.
Bloch, K. (1953). *Helv. chim. acta*, **36**, 1611.
Bradley, J. E. S., Holloway, R. C. & McFarlane, A. S. (1954). *Biochem. J.* **57**, 192.
Cardwell, H. M. E., Cornforth, J. W., Duff, S. R., Holtermann, H. & Robinson, R. (1951). *Chem. & Ind.* p. 389.
Cornforth, J. W. (1954). *Rev. Pure appl. Chem.* **4**, 275.
Cornforth, J. W., Hunter, G. D. & Popjak, G. (1953*a*). *Biochem. J.* **54**, 590.
Cornforth, J. W., Hunter, G. D. & Popjak, G. (1953*b*). *Biochem. J.* **54**, 597.
Cornforth, J. W. & Popjak, G. (1954). *Biochem. J.* **58**, 403.
Cornforth, J. W., Popjak, G. & Gore, I. Youhotsky (1956). *Biochemical Problems of Lipids*, p. 216. Ed. by Popjak, G. & Lebreton, E. London: Butterworths Scientific Publications.
Cornforth, J. W., Youhotsky, I. & Popjak, G. (1954). *Nature, Lond.*, **173**, 536.
Darzens, G. & Levy, A. (1937). *C.R. Acad. Sci., Paris*, **204**, 272.
Dauben, W. G. & Takemura, K. H. (1953). *J. Amer. chem. Soc.* **75**, 6302.
Fieser, L. F., Herz, J. E. & Huang, W.-Y. (1951). *J. Amer. chem. Soc.* **73**, 2397.
Heilbron, I. M. & Wilkinson, D. G. (1932). *J. chem. Soc.* p. 1708.
Heusser, H., Beriger, E., Anliker, R., Jeger, O. & Ruzicka, L. (1953). *Helv. chim. acta*, **36**, 1918.
Heusser, H., Heusler, K., Eichenberger, K., Honegger, C. G. & Jeger, O. (1952). *Helv. chim. acta*, **35**, 295.
Hunter, G. D. & Popjak, G. (1951). *Biochem. J.* **50**, 163.
James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
Laucht, F. (1935). *Hoppe-Seyl. Z.* **237**, 236.
Little, H. N. & Bloch, K. (1950). *J. biol. Chem.* **183**, 33.
Popjak, G. (1950). *Biochem. J.* **46**, 560.

Popjak, G. (1955). *Royal Institute of Chemistry Lectures, Monographs and Reports*, no. 2, p. 17.
Riniker, B., Arigoni, D. & Jeger, O. (1954). *Helv. chim. acta*, **37**, 546.
Ruyle, W. V., Chamberlin, E. M., Chemerda, J. M., Sita, G. E., Aliminosa, L. M. & Erickson, R. L. (1952). *J. Amer. chem. Soc.* **74**, 5929.
Schenck, F., Buchholz, K. & Wiese, O. (1936). *Ber. dtsch. chem. Ges.* **69**, 2703.
Weedon, B. C. L. (1952). *Quart. Rev. chem. Soc., Lond.*, **6**, 380.
Wieland, H., Rath, F. & Benend, W. (1941). *Liebigs Ann.* **548**, 19.
Woodward, R. B. & Bloch, K. (1953). *J. Amer. chem. Soc.* **75**, 2023.
Würsch, J., Huang, R. L. & Bloch, K. (1952). *J. biol. Chem.* **195**, 439.
Zanetti, C. V. (1891). *Gazz. chim. ital.* **21**, II, 168.

The Metabolism of Short-chain Fatty Acids in the Sheep

5. SOME INTERRELATIONSHIPS IN THE METABOLISM OF FATTY ACIDS AND GLUCOSE BY SHEEP-RUMEN EPITHELIAL TISSUE*

By R. J. PENNINGTON and W. H. PFANDER†
Rowett Research Institute, Bucksburn, Aberdeenshire

(*Received* 19 *April* 1956)

Acetic, propionic and butyric acids are produced in large quantities by microbial activity in the sheep rumen. Pennington (1952) showed that each of these acids may be metabolized by the rumen epithelial tissue, which produces ketone bodies from butyrate and, to a smaller extent, from acetate. Rumen epithelium also metabolizes glucose (Pennington & Sutherland, 1956*a*), which, although not normally present in appreciable quantity in the rumen, is available to the epithelial tissue from the blood.

It is of obvious physiological interest to study the metabolism of each one of these compounds in the presence of the others. Several workers have studied such interrelationships in other tissues. Quastel & Wheatley (1933) observed that propionic acid, but not glucose, lowered the production of acetoacetic acid from butyric acid by liver slices. Parnes & Wertheimer (1950) found that the utilization of glucose by rat diaphragm was decreased by acetate if the glucose concentration was not higher than 0·05%. Recent studies on the effects of glucose on the oxidation of fatty acids have given rather conflicting results. Allen, Friedmann & Weinhouse (1955) found that glucose had no appreciable effect on the oxidation of butyrate or palmitate by tissue slices and homogenates. However, by the use of a different technique, Lossow & Chaikoff (1955) observed that glucose spared the oxidation of palmitic acid but that the effect decreased with decrease in chain length of the acid.

In the present work mixtures of these compounds were metabolized *in vitro* by rumen epithelium and the uptake of each substrate and the formation of ketone bodies were measured.

METHODS AND RESULTS

The procedures used in the preparation of the tissue sections, incubation of the tissue with the substrates and the method of ketone-body determination were as previously described (Pennington, 1952). The individual fatty acids in the mixtures were determined by gas–liquid chromatography (James & Martin, 1952). Glucose was determined by the method of Somogyi (1945). Tissue from a single sheep was used in each experiment. The sections were pooled before distributing among the flasks. The duplicate figures in Tables 1 and 3 represent the results obtained with different portions of tissue.

Table 1 shows the results obtained in two experiments in which all the possible combinations of acetate, propionate and butyrate were metabolized.

In spite of relatively large variations in the results, there is good evidence for some effects upon the metabolism of the fatty acids. The uptake of propionate was strongly inhibited by butyrate and that of butyrate inhibited by acetate; both effects were significant at the 1% level. The decrease in acetate uptake caused by propionate, the increase in acetate uptake in the presence of butyrate and the increased butyrate uptake when propionate was present were significant only at the 5% level.

The presence of propionate practically abolished ketone-body production from acetate but had only a small and possibly not significant (P approx. 0·1) effect on ketogenesis from butyrate.

* Part 4: Pennington & Sutherland (1956*b*).

† Present address: College of Agriculture, Department of Animal Husbandry, University of Missouri, Columbia, U.S.A.