[CONTRIBUTION FROM THE CHEMICAL LABORATORY, HARVARD UNIVERSITY]

Cholesterol and Companions. III. Cholestanol, Lathosterol and Ketone 104

BY LOUIS F. FIESER

RECEIVED FEBRUARY 20, 1953

A search for precursors of the two unidentified products of oxidation reported in paper I led instead to isolation of cholestanol, the new companion lathosterol, identified as Δ^7-cholestenol, and a substance $C_{27}H_{44}O_3$ temporarily designated ketone 104, which appears to be an oxidation product of an as yet unknown companion. Cholestanol and lathosterol were isolated by chromatography from enriched mother liquor fractions obtained by a process of quick crystallization of the sterol–acetic acid complex. Like other Δ^7-stenols, lathosteryl acetate is oxidized by chromic acid to the 8α,14α- and 8α,9α-oxido-7-ketones, which have been found reducible with zinc and acetic acid to the corresponding α,β-unsaturated ketones. Oxidation with selenium dioxide at 0–25° constitutes a highly sensitive test by which 2% of material can be detected in a 1-mg. sample of cholesterol; the test is specific to 5α- or Δ^5-steroids having a double bond adjacent to C_{14}. A simple method for isolation of ketone 104 and cholestanone involves a novel phase-separation method for working up a dichromate oxidation mixture and a separation based on the fact that ketone 104 is less reactive than cholestanone to Girard's reagent. Purification of cholesterol through the dibromide removes all known companions except the precursor of ketone 104.

Paper I of this series reported isolation in minute amounts of two products of the oxidation of cholesterol that appeared not to be derived from this substance itself or from 7-dehydrocholesterol or cholestanol, the only known companions. Windaus and Stange[1] isolated 7-dehydrocholesterol by chromatographic fractionation of 2 kg. of egg yolk cholesterol containing 0.18% provitamin by spectrophotometric analysis in a series of elutions utilizing over one thousand liters of solvent. The evidence for the presence of cholestanol is less direct. Schoenheimer[2] isolated cholestanol from gallstone cholesterol, but only after a crude stanol preparation had been equilibrated with sodium in a high-boiling solvent, a method that Windaus and Uibrig[3] had used to demonstrate the presence of cholestanol in the coprostanol from feces. Thus the development of a fully unambiguous method for isolation of cholestanol was a secondary objective of the present search for precursors of the unidentified oxidation products.

Cholestanol seemed a suitable model substance for evaluation of methods of fractionation, particularly since Schoenheimer[2] showed that 6% of cholestanol in cholesterol is not separable by repeated crystallization from alcohol. Acetic acid appealed to me as a promising solvent because of the high solubility of sterol in the hot solvent and the sparing solubility of the 1:1 acetic acid complex that separates on cooling; the complex was first described by Hoppe–Seyler in 1863.[4] Furthermore, structural and steric factors play such an important role in determining the solubilities of digitonides and of the episterol–ketone complexes described in paper I that it seemed reasonable to expect greater differentiation between sterol–acetic acid complexes than between the sterols themselves. Indeed experiment showed that the solubility of cholesterol in acetic acid at 25° is 0.62 g./100 cc., whereas the solubility of cholestanol is 0.90 g./100 cc. Furthermore, a mixture containing 6% cholestanol on one crystallization from acetic acid gave cholesterol in which the presence of a contaminant was no longer detectable from the melting point or rotation. A difficulty associated with crystallization from acetic acid is that esterification occurs very rapidly in the hot solution. Thus cholesteryl acetate was obtained in 92% yield by merely refluxing the sterol in pure acetic acid for 22 hr. The possibility of a selectivity in the reaction was explored, but cholestanol and epicholestanol in the same period of refluxing were found to be esterified to the same extent as cholesterol. In spite of the rapidity of acetylation, a crystallizate containing only a trace of acetate can be obtained by pouring boiling acetic acid (8 cc./g.) onto cholesterol, stirring for about two minutes to effect solution, and stirring very vigorously in an ice-bath until a temperature of 25° is reached. Lots up to 150 g. were crystallized by this method. The acetate that is formed is almost all retained in the mother liquor, since the solubility is 3.25 g./100 cc. The mother liquor material has to be saponified before recrystallization, but the crystallizate requires saponification only if it is to be explored for the presence of a trace substance.

An indication of what might be accomplished by fractional crystallization of cholesterol from acetic acid was obtained by a systematic series of crystallizations of an initial 200 g. of Wilson Co. material (spinal cord and brain of cattle), each time from 8 cc./g. of solvent and after cooling to 25° The first crop, designated A, was washed with methanol to facilitate elimination of acetic acid from the complex, dried to constant weight and recrystallized as before to give A_2, A_3, etc. Mother liquor materials from A_1, A_2, A_3, etc., were recovered, saponified, dried and crystallized from 8 cc./g. of acetic acid to give B_1, B_2, B_3, etc. The mother liquors from the B crops on similar processing afforded C crops; those in turn yielded crops designated D. Twenty-two crystallizations were made and the weights of the different crops determined at representative intervals, with the results plotted in Fig. 1. In the initial crystallization the recovery in the first crop (A_1) is 89%; after about ten crystallizations the recovery reaches a constant value of about 94%. The curve for the second crop rises even more sharply and reaches constancy after 10–15 crystallizations, and it is evident that beyond this point crystallization effects no further change in composition. Fractions A_1 and A_{22}, after precautionary saponification, were indistinguishable in rotation and in melting point (149.5–150.0°). A melting point determination of cholesterol is

(1) A. Windaus and O. Stange, *Z. physiol. Chem.*, **244**, 218 (1936).
(2) R. Schoenheimer, *ibid.*, **192**, 86 (1930).
(3) A. Windaus and Cl. Uibrig, *Ber.*, **48**, 857 (1915).
(4) F. Hoppe-Seyler, *Jahresberichte*, 545 (1863).

valid only if taken in an evacuated capillary tube; air oxidation occurs so readily that a sample heated slowly in an open tube can be caused to melt 3–4° below the true melting point.

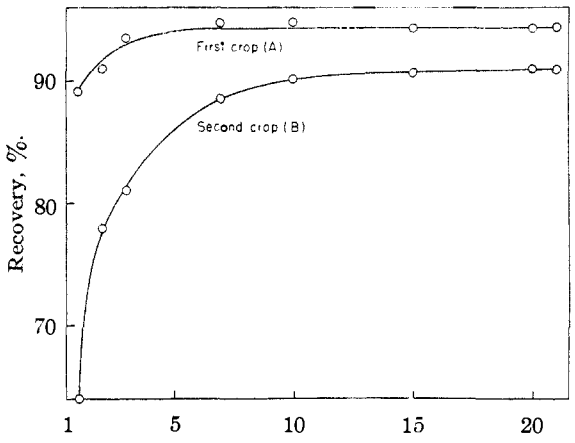


Fig. 1.—Crystallization of cholesterol as the acetic acid complex.

Crops C and D from the first two or three crystallizations seemed to offer the most promise for isolation of companions, but the yields from 200 g. of cholesterol were low: $C_1 + C_2 = 5.5$ g.; $D_1 + D_2 = 3.4$ g., and these concentrates would of course contain the bulk of non-sterol contaminants. However, Dr. E. B. Hershberg kindly had 40 kg. of Wilson cholesterol processed in the Schering Corp. plant and supplied me with large quantities of crude A_1, B_1 and C_1 material. Quick chilling of the hot acetic acid solutions could not be accomplished satisfactorily on a large scale and all fractions had suffered extensive acetylation; nevertheless saponification and further processing gave abundant amounts of C_1 and D_1 material evidently highly enriched in companion substances. After a number of fruitless trials, a first companion was isolated from the mother liquor of the bromination of D_1-acetate and it proved to be cholestanyl acetate. The bromination, attended with copious evolution of hydrogen bromide, afforded an amount of cholesteryl acetate dibromide corresponding to the presence of 36% of cholesteryl acetate.

A sample of D_1-acetate was then chromatographed on twice the usual amount of alumina and separated into 46 fractions, 37 of which appeared to consist largely of mixtures of the acetates of cholesterol and cholestanol. Five terminal fractions contained a different, single component identical with Δ^7-cholestenyl acetate. A new companion was thus identified as Δ^7-cholestenol. In presenting a communication[5] on the isolation of this companion it seemed to me desirable to designate the sterol of biological occurrence by a specific name and I suggested the name lathosterol (Gr. *latho-*, undetected). This name has since been accepted by a number of steroid biochemists and I have used it in this paper. Lathosterol was isolated a second time by fractional crystallization of the 2:1 sterol–oxalic acid complex[6] prepared from D_1 material and chromatographic processing of the acetate derived tiom tail fractions. Subsequent to my communicafron, Idler and Baumann[7] reported the interesting finding that the skins of certain animals contain very large amounts of lathosterol and 7-dehydrocholesterol.

Chemical characterization of Δ^7-cholestenol (synthetic) was made as follows. Oxidation of the stenol acetate Ia with chromic acid in acetic acid gave an easily separable mixture of two ketoxides (II and III) analogous to those obtained by Stavely and Bollenback by similar oxidation of the acetates of 5-dihydroergosterol[8] and of the Δ^7-stenol "α"-spinasterol.[9] The more soluble isomer II corresponded in properties with a ketoxide obtained by Wintersteiner and Moore both by oxidation of $\Delta^{8(14)}$-cholestenyl acetate[10] and by chromic acid oxidation of an oxido alcohol resulting from the action of perbenzoic acid on Δ^7-cholestenyl acetate.[11] A comparison of samples kindly made in Dr. Wintersteiner's laboratory established identity of my product II with theirs. That the isomeric product is the 8,9-oxido-7-ketone (III) can be inferred from the M_D relationships and is conclusively established by conversion of II and III to the common dienone, to be reported later. That the 8,14- and 8,9-oxido bridges are α-oriented is demonstrated by evidence presented in paper IV. A novel reaction, discovered independently by Heusser, Saucy, Anliker and Jeger[12] in another series, is reduction of the ketoxides II and III with zinc dust in acetic acid to the corresponding α,β-unsaturated ketones V and VI. Isomer V was compared directly with material described by Wintersteiner and Moore[11] and found to be identical with their sample.

Buser[13] showed that free Δ^7-cholestenol (I) on either Oppenauer or chromic acid oxidation is converted into the corresponding ketone (IV). This substance is also the initial product of oxidation with dichromate at 25°; further oxidation by the same reagent produces the 3,7-diketo-8α,9α-oxide VII, identical with material prepared by my associate Dr. Wei-Yuan Huang from III by saponification and oxidation. Various oxidizing agents are known to effect dehydrogenation of Δ^7-stenols to $\Delta^{7,9(11)}$-diene, as summarized in paper IV. An addition to the list is N-bromosuccinimide, which converts Δ^7-cholestenyl acetate to the diene VIII.

Of particular significance is the observation that Δ^7-cholestenol, as such or as the acetate, is so sensitive to oxidation that it is attacked by selenium dioxide in benzene–acetic acid rapidly even at 0°, whereas cholesterol is oxidized by the reagent only at 55–60°. Occurrence of a reaction is clearly evident from the appearance of a yellow coloration due to colloidal selenium followed by separation of a red precipitate of selenium. By a micro test

(5) L. F. Fieser, THIS JOURNAL, **73**, 5007 (1951).

(6) J. Mauthner and W. Suida, *Monatsh.*, **24**, 648 (1903); K. Miescher and H. Kägi, *Helv. Chim. Acta*, **24**, 986 (1941).

(7) D. R. Idler and C. A. Baumann, *J. Biol. Chem.*, **195**, 623 (1952).

(8) H. E. Stavely and G. N. Bollenback, THIS JOURNAL, **65**, 1290 (1943).

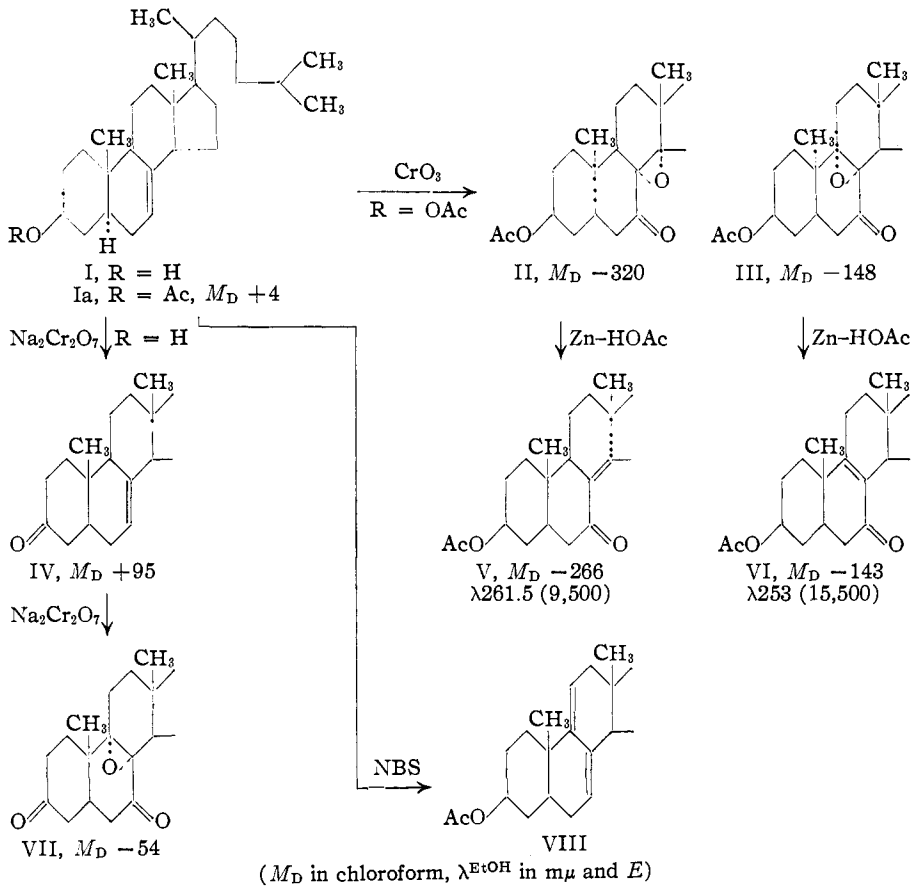
(9) H. E. Stavely and G. N. Bollenback, *ibid.*, **65**, 1600 (1943).

(10) O. Wintersteiner and M. Moore, *ibid.*, **65**, 1513 (1943).

(11) O. Wintersteiner and M. Moore, *ibid.*, **65**, 1507 (1943).

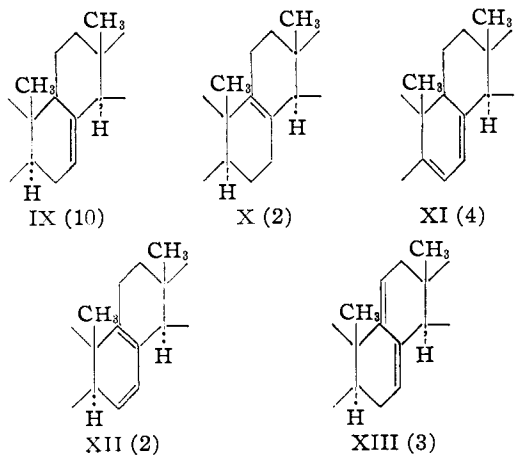
(12) H. Heusser, G. Saucy, R. Anliker and O. Jeger, *Helv. Chim. Acta*, **35**, 2090 (1952).

(13) W. Buser, *ibid.*, **30**, 1379 (1947).



(M_D in chloroform, λ^{EtOH} in mμ and E)

described in the experimental part it is possible, with 1-mg. samples, to differentiate between gallstone cholesterol, which contains about 2% of lathosterol, and the same material purified through the dibromide, a process that completely eliminates lathosterol by converting it to products of dehydrogenation. Tests of a number of unsaturated steroids available in this Laboratory, supplemented by tests made with Drs. George Rosenkranz and Carl Djerassi at the Syntex laboratory and another series kindly made by Dr. Derek H. R. Barton in London, show that low temperature (25°) oxidation by selenium dioxide is specific to steroids of the allo- or Δ^5-series with a double bond adjacent to the 14α-hydrogen atom, as in Δ^7- and Δ^8-stenols (IX, X) and in $\Delta^{5,7}$-, $\Delta^{6,8}$- and $\Delta^{7,9(11)}$-dienes (XI–XIII). The number of compounds tested is given in parentheses after the formula numerals. The investigation reported in paper IV indicates that initial attack by the reagent is probably at the activated 14-position. That ergosterol (XI) reacts readily with selenium dioxide was reported by Montignie[14] in a publication three months before the Riley paper introducing general use of the reagent[15]; Montignie also showed that the reaction (in ethanol at steam-bath temperature) can be conducted on a micro scale for differentiation between ergosterol and cholesterol.

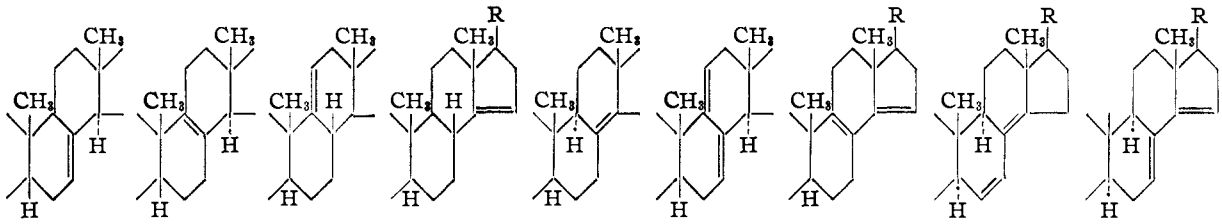


Structural types with which the present test is negative are listed in formulas XIV–XXII. The negative-testing enes XIV and XV and the diene XIX correspond to the positive-acting types IX, X and XIII except that they are inverted at C_5. The relationship suggests that the oxidation involves attack from the rear and that in the A/B-*cis* configuration ring A is folded back to form a cage rendering C_{14} inaccessible. The negative response of XVI and XVII shows that an activated β-hydrogen atom at C_8 is not vulnerable to attack, and the behavior of XVIII, XXI and XXII shows that an activated α-hydrogen atom at C_9 does not share the sensitivity of one at C_{14}.

The isolation of lathosterol did not solve the problem that initiated this investigation, for the monoketone IV and the diketoöxide VII derived from this sterol differ from the substances $C_{27}H_{46}O$ and $C_{27}H_{42}O_3$ obtained from cholesterol. Consequently a search for still another companion was undertaken. By this time it was evident that the separation of either lathosterol or cholestanol from cholesterol by processes of crystallization and chromatography had been rendered particularly difficult by the presence of both these companions in all enriched fractions. Rather than face the prospect of processing a four-component system, I tried various schemes for elimination of one or more known components. Low temperature oxidation with selenium dioxide is so highly specific that it seemed safe to assume that the unknown companion would be as inert to the reagent as cholesterol and cholestanol are. Lathosteryl acetate is oxidized completely to a yellowish mixture

(14) E. Montignie, *Bull. soc. chim.*, **51**, 144 (1932).

(15) H. L. Riley, J. F. Morley and N. A. C. Friend, *J. Chem. Soc.*, 1875 (1932).



XIV (3) XV (1) XVI 5α or β(3) XVII 5α or β(3) XVIII 5α or β(5) XIX (2) XX 5α or β(3) XXI (2) XXII (1)

of products that are all strongly adsorbed on alumina. When a mixture of cholesteryl acetate (purified through cholesterol dibromide) and 17% of lathosteryl acetate was oxidized with selenium dioxide and chromatographed, 91% of the cholesteryl acetate was recovered in the petroleum ether eluate, and benzene then eluted a yellow oil corresponding exactly in weight to the lathosteryl acetate taken. Although the method thus appeared valid, attempts to apply it to the processing of various fractions failed to disclose any new companion.

The next plan was to eliminate cholesterol by dichromate oxidation under conditions known (paper II) to effect complete conversion of the Δ^5-stenol to either acidic products or to Δ^4-cholestene-3,6-dione and to remove these by extraction with soda and then with Claisen alkali, respectively. Trial with a mixture of purified cholesterol with 5% each of cholestanol and lathosterol gave results even better than expected; the cholesterol was effectively removed and the small residue from extraction with Claisen alkali on chromatography gave a series of early fractions consisting of cholestanone uncontaminated with lathostenone and amounting to a recovery of 74%. The lathosterol evidently had all been converted either into acids or into the strongly adsorbed diketoöxide VII. It remained to be seen if any other companion substance would withstand the oxidation and yield an isolable product. When A_1 cholesterol was put through the process of oxidation, extraction and chromatography, the early petroleum ether eluates afforded a very small amount of cholestanone and this was followed by a new substance that is beautifully crystalline and melts sharply at 123.5–124.5°, α_D −36.6° Chf, λ^{Chf} 5.81 μ. This substance is temporarily designated ketone 104 (notebook page). It was isolated on numerous subsequent experiments and four closely agreeing analyses of three separate samples indicate the formula $C_{27}H_{44}O_3$. Beyond the observations that the ketone is saturated to tetranitromethane and stable to refluxing with zinc and acetic acid or with ethanolic hydrochloric acid, I have delegated further characterization to my associate Dr. Bhattacharyya.

In 20-g. oxidations A_1 and A_2 cholesterol gave 40–47 mg. of ketone 104; A_6 gave 107 mg.; B_1–B_4 fractions gave 14–25 mg. The results suggest that the precursor of ketone 104 concentrates slowly in the acetic acid crystallizate, but the differences may be due merely to differences in the amount of cholestanone from which separation has to be made. Discovery that the precursor is precipitated along with cholesterol dibromide greatly simplified the problem of isolation, since purification through the dibromide completely eliminates cholestanol. Although the process of purification is attended with some 32% loss, the over-all yield *via* the dibromide is about the same as in a direct oxidation. Yields of ketone 104 from various dibromide-purified samples were as follows: Wilson Co. material, lots of increasing age: 85, 90, 123, 139 mg.; shark- and skipjack-liver oil sterol, 106, 110 mg.

With use of this method of approximate oxidative analysis of 20-g. samples as a guide, I explored fractionation by selective bromination, triangular crystallization of the acetate dibromide (344 fractions); repeated bromination and regeneration; fractionation of Δ^5-cholestene-3-one; and countercurrent distribution in 2-l. separatory funnels. The results were not very promising, and it seemed best to defer further trials for a time in the hope that the analytical method could be adapted to a microscale. Adaptation to large-scale preparation was a further objective. Progress in both directions resulted from the observation that ketone 104, although it reacts with ketonic reagents, is distinctly less reactive than cholestanone. On treatment with Girard's reagent T in methanol–acetic acid at room temperature for one hour, cholestanone reacts completely and ketone 104 does not react at all, and a sharp separation of the two ketonic components of the Claisen alkali extraction residue is thus effected very easily; chromatography is required for full purification of ketone 104.

A further simplification was developed as follows. In the standard procedure the oxidation mixture was diluted with water, extracted with ether, the benzene–ether solution evaporated and the residue dissolved in hexane for extraction with Claisen alkali. I tried introduction of hexane at various earlier stages and then found that if the oxidation is done in the usual way in 1:2 benzene–acetic acid and then, at the end, 1 volume of petroleum ether (or hexane) is added this causes clean phase separation. The lower layer, comprising about one-third of the total fluids, is rich in acetic acid, chromium compounds, and acidic oxidation products and can be drawn off and discarded. Addition to the upper phase of a small volume of water (50 cc.) causes separation of a further large lower layer (250 cc.), and by a few repetitions of the process a faintly yellow petroleum ether–benzene solution is obtained directly suitable for extraction of the enedione by Claisen alkali. This procedure greatly reduces the working time and cuts in half the total volume of fluids. Another simplification consists in conducting the oxidation not at a temperature optimal for formation of Δ^4-cholestene-3,6-dione

(9–15°), but at a temperature optimal for further oxidation of this substance to acids, which are largely removed in the chromate liquor; the otherwise tedious extraction with Claisen alkali is thus greatly facilitated.

Further improvements both in the large-scale production of ketone 104 and in the direction of microanalysis will be reported in papers with Dr. Bidyut Bhattacharyya and Dr. Wei-Yuan Huang, respectively.

Discussion.—The present evidence indicates that cholesterol from spinal cord and brain of cattle contains lathosterol, cholestanol and probably a precursor of ketone 104. Treatment as acetate and chromatography removes lathosterol (and any 7-dehydrocholesterol) and purification through the dibromide gives an as yet inseparable mixture of cholesterol and about 0.5% of pro-ketone 104. It is now evident that interpretation of data on the biological effects of cholesterol and on the radioactivity of biosynthetic material from active precursors will be subject to uncertainty until the contributions of the companion substances can be evaluated.

An interesting biogenetic relationship is that lathosterol is the 5,6-dihydro derivative of 7-dehydrocholesterol just as cholestanol is the 5,6-dihydride of cholesterol. The relative amounts, however, are reversed: cholestanol is a trace substance in comparison to cholesterol, whereas the amount of lathosterol present outshadows the amount of the provitamin.

Acknowledgments.—In addition to the acknowledgments of paper I, I wish to thank Dr. E. B. Hershberg of Schering Corp., Drs. George Rosenkranz and Carl Djerassi of Syntex S.A., Dr. Oskar Wintersteiner of the Squibb Institute and Dr. Derek H. R. Barton of Birkbeck College for the contributions noted, and Dr. Max Tishler of Merck and Co., Inc., for help of various kinds.

Experimental

Wilson Co. Cholesterol.—Dr. David Klein of the Wilson Co. has told me that the Wilson cholesterol has invariably been extracted after saponification of a standard blend of spinal cord and brain of cattle. The present experiments were done over a period of about two years with a variety of lots of Wilson material not now identifiable, but no variation from lot to lot was observed. Typical material had a urine odor which became very faint when the cholesterol was air dried at 90° for 60 hr.; no loss in weight was detected in this period. Typical constants found were: m.p. 147–148.5, 146.5–148°, $\alpha^{25}D$ −39° Chf (*c* 2.68).

Crystallization from Acetic Acid.—Crystallization of the 1:1 cholesterol–acetic acid complex must be done rapidly if extensive acetylation is to be avoided. The following procedure was used for the preparation of large batches of mother liquor concentrate. Nine hundred g. of Wilson cholesterol was crystallized in 6 lots of 150 g., each from 1.2 l. of acetic acid. The cholesterol was placed in a beaker, the acetic acid was heated to the boiling point in an Erlenmeyer flask and poured in and the mixture was stirred vigorously with a wooden paddle until the solid had all dissolved (about 2 min.). The beaker was then plunged into a large ice-bath and stirred vigorously and the complex dislodged from the walls with the paddle; with efficient hand stirring and cooling to overcome the surge of heat of crystallization, the temperature can be brought to 25° while the next portion of solvent is being brought to the boil. The complex was then collected on a large Büchner funnel, rinsed in with acetic acid, and the cake pressed down and sucked well. When the material was to be recrystallized, the cake was transferred while still moist with acetic acid. The method of drying the complex is described below. The combined mother liquors were diluted extensively with water in large round-bottomed flasks and the fine suspension let stand until the solid had collected in a thin layer at the top. The clear liquor was then removed by siphon and the material collected by ether extraction. This material is a mixture of sterols and sterol acetates. In the 900-g. run cited the fraction was acetylated and afforded 42.5 g. of sterol acetate.

The data reported in Fig. 1 were obtained by similar crystallization of 200 g. of Wilson cholesterol (8 cc. acetic acid./g.). After the complex had been washed with acetic acid (200 cc.) and sucked well, it was washed further with 250 cc. of methanol, which greatly facilitated drying. The solid, designated A_1, was air dried overnight at 25° and then to constant weight at 90°; yield 178.4 g. (solvent-free) m.p. 149–150°. This was recrystallized to give A_2 and A_3; A_3 was then recrystallized four times without drying fractions 4, 5, 6 but each time with an estimated 8 cc./g. of solvent.

The combined mother liquor and methanol wash from A_1 was diluted with 2 l. of water, let settle, the bulk of the liquor removed by siphon, and the solid collected on a Büchner. The aqueous mother liquor on extraction with ether gave 0.99 g. of a yellow glass (E). The precipitate, a sticky solid (18.4 g.) was crystallized as above from 145 cc. of acetic acid to give fraction B_1; 9.27 g., m.p. 149–150°. The mother liquor was diluted and the material recovered by ether extraction, saponified, and the brown glass dissolved in 100 cc. of acetic acid. After some cooling and scratching, a colorless complex separated, and when washed with methanol and dried afforded 2.85 g. of fraction C_1, m.p. 138–140°. The final mother liquor was processed as before and the ether extract washed with alkali, which discharged a good bit of color. The residue (D_1) was a brown glass weighing 2.38 g.

The total recovery in this series was 96.9%; in all subsequent crystallizations it was 98.4–99.1%. The per cent. recovery of second-crop material (B) was calculated from the weight of B in comparison with the total sample taken less the weight of A.

Melting Point.—Samples of A_1 and A_{22} material were submitted to saponification (which did not change the m.p.); crystallized from methanol, and dried at 30° for 15 hr. In parallel determinations done very slowly in evacuated capillaries, both samples melted at 149.5–150.0° and remelted at 150–150.5°. The infrared spectra and specific rotations were indistinguishable: $\alpha^{21}D$ −39.6, −39.9° Chf (*c* 1.49, 1.73), −35.0, −35.0° Di (*c* 1.11, 1.52).

When the melting point of material purified as above or through the dibromide was observed in an open capillary (soft glass) a distinct depression was observed as the result of air oxidation. For example, when heated in a bath at 142–145° A_1 material soon became soft and in 10–15 min. formed a clear melt (at 145°); A_{22} after 1 hr. remained unmelted but was soft and yellowish at the top. When A_{22} was heated in an oven at 80° for 15 and 37 hr., the m.p. was 148–148.5° and 147.5–148.5°. A sample heated in an evacuated capillary at 100° for 15 hr. melted at 149.5–150.0°.

Solubility in Acetic Acid.—Solubilities determined by crystallizing material from acetic acid as above, collecting the crystallizate after thorough stirring at 25°, and drying to constant weight were as follows: cholesterol (A_{10-15}) 0.62 g./100 cc., cholesteryl acetate 3.25 g./100 cc., cholestanol 0.90 g./100 cc. Mixtures of A_1 cholesterol with cholestanol were crystallized once from methanol and characterized. A 2% mixture had m.p. 148.7°, αD −38.8° Chf; a mixture containing 6.2% cholestanol melted at 146–147°, αD −34.2° Chf. The second mixture was crystallized once from 8 cc. of acetic acid per g. (25°) and then from methanol, when the m.p. (149.5–150.0°) and rotation (−40° Chf) no longer showed the effect of a contaminant.

Acetylation and Deacetylation.—A solution of 5 g. of A_1 cholesterol in 35 cc. of acetic acid was refluxed for 22 hr. and let cool. A first crop of pure cholesteryl acetate separated (4.43 g.), m.p. 115–116°, αD −42.5° Chf (*c* 2.75). Dilution of the mother liquor with methanol gave 0.14 g. of white solid, m.p. 114–115°, and dilution of the yellow mother liquor with water and crystallization from ethanol gave 0.33 g., m.p. 114–115°, and 0.18 g., m.p. 109–111°; total yield 5.08 g. (92%).

Refluxing of 5 g. of cholestanol in 30 cc. of acetic acid for 22 hr. afforded a total of 5.13 g. (92%) of cholestanyl ace-

tate. The main crop (4.94 g.) melted at 110–111° and the m.p. was unchanged on crystallization from methanol. Epicholestanol (0.8 g., m.p. 187–188°) on similar treatment and crystallization of the product from methanol afforded the acetate in two crops: 0.60 g., m.p. 95.5–96°; 0.11 g., m.p. 95–96°.

The standard preparative procedure for acetylation was to heat a mixture of 150 g. of cholesterol, 300 cc. of pyridine and 150 cc. of acetic anhydride for one-half hr. on the steam-bath, dilute extensively with water, collect the granular solid and wash it well; yield of fully dry acetate, 166 g. (100%). Saponification was effected by dissolving the acetate in the minimum amount of 95% ethanol, adding 25% aqueous sodium hydroxide or Claisen alkali until the solution contained excess alkali and remained alkaline after heating for 10 min. on the steam-bath. The same process was used for saponification of traces of acetate present in cholesterol that had been crystallized from acetic acid. In this case only 5 cc./g. of 95% ethanol is required to effect solution at the boiling point, whereas 35–40 cc./g. of methanol is required. Deacetylation of acetic acid-crystallized material by refluxing in methanol with boron fluoride etherate (0.005 cc./cc. of methanol) is satisfactory but requires an excessive amount of solvent. The solubility of cholesterol in methanol at room temperature is approximately 0.0054 g./cc.

Isolation of Cholestanol.—Cholestanol and lathosterol were first isolated from a mother liquor concentrate derived from a crystallization of 40 kg. of Wilson cholesterol from acetic acid conducted in the Schering Corp. plant through the courtesy of Dr. E. B. Hershberg. The aim was to follow the laboratory procedure given above, but the large volumes of hot solution could not be chilled rapidly enough and all fractions were found to be extensively acetylated. Hence the differentiation between fractions A_1 (33.5 kg.), B_1 (5.4 kg.) and C_1 (solvent-moist) was much less distinct than in the small laboratory-processed batches. Nevertheless, the crude fraction C_1 afforded an abundant source of material considerably enriched in companion substances. Portions were saponified, and when the sterol (m.p. 142–144.5°) was crystallized quickly from acetic acid (8 cc./g.) it gave "Schering D_1" material, m.p. 130–135°. The mother liquor from crystallization of D_1 was chromatographed as the acetate but no pure products were isolated. The first four fractions (m.p. 88–100°) were combined, saponified (m.p. 132–135°) and brominated, when 23% separated as dibromide; chromatography of the mother liquor afforded a little cholestanol, m.p. and mixed m.p. 143–144°.

Very slow crystallization of Schering D_1 acetate from acetic acid (4 cc./g.) by the triangulation scheme seemed to effect little separation. The head and tail fractions melted at 91–94° and 98–100°, and from the amount of cholesteryl acetate dibromide obtained (from 2 g.) in comparison with the yield from cholesteryl acetate, the amount of the latter appeared to be close to 36% in each case. The mother liquors from the two brominations were combined (3 g.) and chromatographed; petroleum ether eluted first a yellow pigment and a series of fractions that on crystallization from methanol afforded 270 mg. of pure **cholestanyl acetate**, as long flat needles, m.p. and mixed m.p. 110–111°, and 0.63 g. of crude material.

Anal. Calcd. for $C_{29}H_{50}O_2$ (430.69): C, 80.88; H, 11.71. Found: C, 80.75; H, 11.65.

Isolation of Lathosterol.—Two grams of Schering D_1 acetate was adsorbed onto 100 g. of alumina and eluted with 90-cc. portions of petroleum ether over a period of 3 days; nothing was eluted in the first 2 hr. Each fraction was crystallized from about 4.5 cc. of methanol, similar fractions combined and second and third crops obtained from combined mother liquors. Fractions 1–12 (529 mg.) all melted in the range 103–104.5°, were Liebermann–Burchard positive, and bromination of first crop material indicated the presence of 36% cholesteryl acetate. The other major component undoubtedly is cholestanyl acetate. Synthetic 1:2, 1:1 and 2:1 mixtures of the stenyl and stanyl acetates were crystallized from methanol and found to melt at 104.5–105.0°, 103.5–105.0° and 103–104°. Fractions 12–23 (443 mg.), melting in the range 99–103.5°, also appeared to consist largely of cholesteryl and cholestanyl acetates; bromination indicated the presence of 39% of cholesteryl acetate. Fractions 24–37, m.p. 97.5–99°, α_D −16.0° Chf (fraction 26), −14.6° Chf (fractions 35–37) were comparable; the optical rotation is that calculated for a 1:1 mixture of cholesteryl and cholestanyl acetates. Fractions 38–41 (50 mg.) appeared intermediate and were discarded.

The next 5 fractions, eluted by 9:1 petroleum ether–benzene, all melted at 118–119° and afforded a total of 123 mg. of **lathosteryl acetate**. Recrystallization of the first crop material gave colorless plates, m.p. 118–119°, $\alpha^{22}D$ +1.5° Chf (*c* 2.05). A mixture with Δ^7-cholestenyl acetate (m.p. 118–119°, α_D +2.4° Chf, +9.4° Di) melted at 119–120°.

Anal. Calcd. for $C_{29}H_{48}O_2$ (428.67): C, 81.25; H, 11.29. Found: C, 81.07; H, 11.50.

Saponification and crystallization from methanol afforded free **lathosterol**, m.p. 125–126°, $\alpha^{27}D$ +5.7° Chf (*c* 1.95). A mixture with Δ^7-cholestenol (m.p. 125–126°, α_D +1.5° Chf, +10.0° Di) melted at 125–126° (evacuated capillaries).

Anal. Calcd. for $C_{27}H_{46}O$ (386.64): C, 83.87; H, 11.99. Found: C, 83.70; H, 12.15.

The total material eluted from the column was 465 mg. less than that adsorbed, but terminal stripping of the column eluted no further material.

Lathosteryl acetate was isolated a second time in the course of one of several unsuccessful attempts to isolate a further companion by fractionation of the 2:1 sterol–oxalic acid complex.[7] In a typical experiment the complex was prepared by slow addition of a solution of 24 g. of anhydrous oxalic acid in 400 cc. of ether (absolute) to a solution of 200 g. of Wilson cholesterol in 1.2 l. of ether. Crop 1 (182.9 g.) was collected after cooling at 5° and six further crystalline crops were obtained after successive concentration. Each crop, or a sample of it, was converted to sterol by dissolving 10 g. of complex in 100–150 cc. of acetone, adding 150 cc. of water and collecting the precipitated sterol. The total recovery was 197.7 g. of free sterol. Analyses by Koji Nakanishi indicated the following content of Δ^7-stenol: crop 1, 0.36, 0.40%; crop 7 (0.8% of total, m.p. 133–138°), 14.50%.

In the successful isolation experiment 33 g. of oxalic acid complex from Schering sterol D_1 was fractionally crystallized from ether by the triangulation scheme into a total of 49 fractions. These were converted to sterol acetate by slurrying the complex (11 g.) with pyridine (30 cc.), adding acetic anhydride (8 cc.) cautiously (heat effect), and heating for 15 min. on the steam-bath. The solution was then cooled, treated with 2.5 cc. of water; after an initial vigorous reaction, excess water was added and the granular acetate collected. About one-third of the material recovered (early fractions) was identified as substantially cholesterol. Four tail fractions totalling 5.6 g. as acetate appeared from the low m.p. (94–98°) to be enriched in companion substances. The acetate was saponified and the sterol crystallized from acetone in a series of four crops. The first crop (1.26 g., m.p. 137.5–138.5°) on acetylation and chromatography afforded, in a series of late fractions, a total of 42 mg. of lathosteryl acetate, m.p. 116–119°; recrystallized, the substance formed plates, m.p. and mixed m.p. 119–120°, $\alpha^{22}D$ +0.8° Chf (*c* 0.96).

$\Delta^{7,9(11)}$-Cholestadienyl Acetate from Δ^7-Cholestenyl Acetate.—A solution of 1 g. of Δ^7-cholestenyl acetate in 10 cc. of ether, 5 cc. of methanol and 1 cc. of pyridine was treated at room temperature with 0.72 g. of N-bromosuccinimide. The solution turned yellow in a few minutes and showed no further change in 2 hr., when it was diluted and extracted with ether. The washed and dried ethereal extract was concentrated to a small volume and the ether then displaced by methanol. The brown solution slowly deposited long slender needles, which when washed with methanol were pure white; yield 0.27 g., m.p. 118–119.5°, mixed m.p. with starting material 105–108°, mixed m.p. with $\Delta^{7,9(11)}$-cholestadienyl acetate 118–119°, λ^{EtOH} 236, 243, 250 mμ (12,600, 14,200, 9,130).

Anal. Calcd. for $C_{29}H_{46}O_2$ (426.66): C, 81.63; H, 10.87. Found: C, 81.47; H, 10.69.

Selenium Dioxide Test.—A 0.1 *M* solution of selenous acid in acetic acid added to a solution of a Δ^7-stenol or its acetate in benzene or toluene effects rapid oxidation at 25°, or even at 0°, with liberation of selenium, detectable first from the yellow color of the colloidal solution and then as a red precipitate. Cholesterol purified through the di-

TABLE I

SELENIUM DIOXIDE TEST

Type	Positive Compound	Type	Negative Compound
5α-Δ^7	Δ^7-Cholestenol; acetate	5β-Δ^7	Methyl Δ^7-lithocholenate
	Δ^7-Cholestene-3-one		$\Delta^{7,22}$-Coproergostadiene-3β-ol acetate,[a] benzoate[a]
	5-Dihydroergosteryl acetate	5β-Δ^8	Methyl Δ^8-lithocholenate 3-acetate
	Δ^7-Ergostene-3-one[a]	5α-$\Delta^{9(11)}$	3β-Acetoxy-$\Delta^{9(11)}$-22-isoallospirostane[b]
	$\Delta^{7,22}$-Ergostadiene-3-one[a]		3β-Acetoxy-$\Delta^{9(11)}$-allopregnene-20-one[b]
	Δ^7-Ergostenyl benzoate[a]	5β-$\Delta^{9(11)}$	Methyl $\Delta^{9(11)}$-lithocholenate 3-acetate
	γ-Diosgenin acetate[b]	5α-Δ^{14}	Δ^{14}-Ergostenyl benzoate[a]
	γ-Allopregnenolone acetate[b]		Δ^{14}-Ergostene-3-one[a]
	$\Delta^{7,16}$-Allopregnadiene-3β-ol acetate[b]	5β-Δ^{14}	Methyl Δ^{14}-lithocholenate 3-cathylate
5α-Δ^8	Dihydroxymosteryl acetate	5α-$\Delta^{8(14)}$	$\Delta^{8(14)}$-Dehydrotigogenin acetate[b]
	Δ^8-Ergostene-3β-ol acetate[a]		$\Delta^{8(14)}$-Ergostenyl acetate[a]
$\Delta^{5,7}$	Ergosterol; acetate		3β-Acetoxy-$\Delta^{8(14),16}$-allopregnadiene[b]
	7-Dehydrocholesterol; acetate	5β-$\Delta^{(8,14)}$	Apocholic acid
$\Delta^{6,8}$	$\Delta^{6,8}$-Cholestadiene-3β-ol acetate,[a] p-nitrobenzoate[a]		Methyl $\Delta^{8(14)}$-lithocholenate 3-acetate
$\Delta^{7,9(11)}$	$\Delta^{7,9(11)}$-Cholestadienyl benzoate	5β-$\Delta^{7,9(11)}$	$\Delta^{7,9(11)}$-Lithocholadienic acid; methyl ester acetate
	$\Delta^{7,9(11)}$-22-Isospirostadiene-3β-ol acetate[b]	5α-$\Delta^{8,14}$	$\Delta^{8,14}$-Ergostadiene-3-one[a]
	$\Delta^{7,9(11)}$-Ergostatetraene-3β-ol benzoate[a]		$\Delta^{8,14}$-Cholestadienyl acetate
?	Westphalen's diol[b]	5β-$\Delta^{8,14}$	Methyl $\Delta^{8,14}$-lithocholadienate 3-acetate
		5α-$\Delta^{6,8(14)}$	$\Delta^{6,8(14)}$-Ergostatriene-3β-ol[a]; acetate[a]
		5α-$\Delta^{7,14}$	Ergosterol-B_8-acetate

[a] Tests conducted by Dr. D. H. R. Barton. [b] Tests conducted at the Syntex laboratories.

bromide is oxidized only at 50–60° and in a test carried out at room temperature the solution remains colorless for several hours. Tests on the unsaturated substances listed in Table I were conducted by dissolving about 1 mg. of sample in 0.5 cc. of benzene and adding at room temperature 1 cc. of a solution prepared by dissolving 1.29 g. of selenous acid or 1.11 g. of selenium dioxide in 2 cc. of water by heating and diluting with acetic acid to a volume of 100 cc. With the compounds listed as positive a yellow color was discernible in 1–8 min. and red selenium was deposited abundantly in 10–20 min. Substances that gave no yellow color in 1 hr. are listed as negative; in some instances a faint yellow color subsequently developed. Additional compounds that gave a negative test are: vitamin D_3, stigmasterol, tigogenin acetate, 3-epismilagenin acetate, $\Delta^{4,6}$-androstadiene-3,17-dione, 1-methyl-Δ^6-dehydroandrosterone acetate, Δ^4-cholestene and methyl Δ^3-cholenate. The formula attributed to Westphalen's diol by Ellis and Petrow[16] is inconsistent with the observation that the diol gives a strong positive test.

Micro Tests.—When the selenium dioxide test is carried out on a microscale in commercial melting point capillaries of uniform size one can, with a little practice, estimate amounts of Δ^7-stenol in cholesterol in the range of 2–20% with considerable accuracy. A sample is introduced and tamped down until the column measures 3 mm., which corresponds to 1 mg. of sterol. With use of a pipet made by drawing down a capillary to a fine tip, benzene is introduced and whipped down until the column is extended to 6 mm.; the tube is heated until solution is complete. Then a 4-mm. column of 0.1 *M* selenous acid solution is introduced at the top of the tube, whipped down, and the tube is placed on a piece of white paper and the time noted. A faint color is most easily detected by viewing the tube in a line deviating just a little from the axis. With this test a sharp differentiation can be made between gallstone cholesterol (2–3% lathosterol) and dibromide-purified cholesterol.

The fast action of Δ^7-stenols in the Liebermann–Burchard test observed by Moore and Baumann[17] provides the basis for a second microtest. A few very small particles of gallstone cholesterol are introduced into a melting point capillary and shaken and tamped down to form a 0.5-mm. layer at the very bottom. A second tube is prepared containing dibromide-purified cholesterol. Then enough chloroform is added to each tube to give a 10-mm. column of solution. The test reagent is made by adding one drop of 95% sulfuric acid to 1 cc. of chilled acetic anhydride. The tubes are held horizontally while a 3-mm. column of test solution is introduced at the open end; then the two tubes are grasped together at the open ends, the solutions whipped down, and the time noted to the nearest second. The tube containing gallstone cholesterol develops a strong color in a few seconds and at a time when the other tube is colorless. Differentiation between dibromide-purified and Wilson cholesterol is possible, but the difference is very subtle.

Δ^7-Cholestene-3-one[13] and 8α,9α-Oxidocholestane-3,7-dione (VII).—A chilled solution of 1.28 g. of sodium dichromate dihydrate in 10 cc. of acetic acid was added to a solution at 5° of 1 g. of Δ^7-cholestenol in 10 cc. of benzene and 20 cc. of acetic acid. The sterol chromate separated and then dissolved in about 1 hr. at 5–9°. After 6 hr. the mixture was worked up. A trace of acid (0.09 g.) was removed and the slightly yellow neutral fraction (0.80 g.) chromatographed. 1:1 Petroleum ether–benzene eluted Δ^7-cholestene-3-one in a series of fractions, the best of which when combined and crystallized from methanol gave 0.50 g. of plates of the pure ketone, m.p. 146–147°. Elution with benzene and crystallization from methanol afforded a few mg. of the oxidoketone, m.p. 179.5–181°, identical with that described below.

For full oxidation, 0.5 g. of Δ^7-cholestene-3-one in 25 cc. of acetic acid was treated at 25° with 1 g. of dichromate in 25 cc. of acetic acid and let stand for 2 days. Chromatography of the neutral material (0.44 g.) and crystallization from methanol gave 38 mg. of monoketone, m.p. 146–147°, and 35 mg. of the oxidodione as long blades, m.p. 180.5–181.5°, $\alpha^{22}D$ −13° Chf; undepressed on admixture with an authentic sample prepared by Dr. Wei-Yuan Huang, m.p. 179–181°, αD −13° Chf.

Anal. Calcd. for $C_{27}H_{42}O_3$ (414.61): C, 78.21; H, 10.21. Found: C, 77.95; H, 10.04.

3β-Acetoxy-8α,9α-oxidocholestane-7-one (III).—A solution of 10 g. of Δ^7-cholestenyl acetate in 500 cc. of acetic acid was let cool to about 30° and treated with part of a solution of 15 g. of chromic anhydride in 10 cc. of water and 20 cc. of acetic acid. The solution was then cooled in ice and the temperature controlled to 25° during addition of the rest of the reagent. After standing overnight the brown solution was diluted with 170 cc. of water, which caused separation of III in crystalline form. The material was collected, washed with water and then methanol, and dried; yield 1 g., m.p. 173–175°. The substance is only moderately soluble in methanol and crystallizes readily in flat blades, m.p. 177.5–178°, αD −32.2 ± 0.3° Chf.

(16) B. Ellis and V. Petrow, *J. Chem. Soc.*, 2246 (1952).

(17) P. R. Moore and C. A. Baumann, *J. Biol. Chem.*, **195**, 615 (1952).

Anal. Calcd. for $C_{29}H_{46}O_6$ (458.66): C, 75.94; H, 10.11. Found: C, 75.75; H, 10.39.

Buser[13] reported an apparently identical oxidation product, m.p. 177–179° (found: C, 75.57; H, 10.09) but did not formulate it correctly. I tried oxidations with a large excess of dichromate at 25° for periods up to 64 hr. and at 42° for 5–15 hr., but some starting material was always recovered (on chromatography) and the results were distinctly inferior to those with aqueous chromic acid in acetic acid. Anhydrous chromic anhydride reacted too vigorously at 25° and at 0° oxidation did not proceed at all.

3β-Acetoxy-8α,14α-oxidocholestane-7-one (II).—Ether extraction of the diluted acetic acid mother liquor of the above oxidation gave 9.1 g. of yellow glass, part of which was chromatographed. No starting material was found and the only crystalline material, encountered in early fractions, was the 8,14-oxide; later fractions were all yellow gums. The solid ketoxide is only moderately soluble in 30–60° petroleum ether and crystallizes in rosettes of glistening needles, m.p. 142–142.5°, α_D −69.8 ± 0.8° Chf. It is very soluble in methanol and crystallizes slowly, as the solvent evaporates, in long needles.

Anal. Calcd. for $C_{29}H_{46}O_4$ (458.66): C, 75.94; H, 10.11. Found: C, 75.80; H, 10.21.

Comparison with a sample prepared by oxidation of $\Delta^{8(14)}$-cholestenyl acetate (m.p. 139.5–140°, α_D −75.7° Chf) was kindly made in Dr. Wintersteiner's laboratory and there was no depression in m.p.

3β-Acetoxy-Δ⁸-cholestene-7-one (VI).—A solution of 852 mg. of the 8,9-ketoxide in 25 cc. of acetic acid was refluxed with 1 g. of zinc dust for 3 hr. and the colorless solution was filtered, diluted with water and the product extracted with ether and crystallized from methanol (moderately soluble). A first crop of long blades weighed 591 mg. (72%), m.p. 154.5–155.5°; second crop, m.p. 140–144°. A recrystallized sample melted at 155–156°, $\alpha^{25}D$ −32.3 ± 0.8° Chf, λ^{EtOH} 253 mμ (15,500), λ^{Chf} 5.84, 6.04, 6.3, 8.0 μ.

Anal. Calcd. for $C_{29}H_{46}O_3$ (442.66): C, 78.68; H, 10.48. Found: C, 78.78; H, 10.50.

3β-Acetoxy-Δ⁸⁽¹⁴⁾-cholestene-7-one (V).—Reduction of 3β-acetoxy-8α,9α-oxidocholestane-7-one was conducted as described for the isomer and the solution filtered and diluted with water, when the product separated as a filterable solid that after crystallization melted at 138–139°, α_D −58° Chf. A better sample was prepared by Wei-Yuan Huang, who from 1 g. of ketoxide obtained 677 mg. (69%) of product m.p. 142–144°, α_D −60 ± 1° Chf, λ^{EtOH} 261.5 mμ (9,500), λ^{Chf} 5.80, 5.98, 6.29, 7.95 μ.

Anal. Calcd. for $C_{29}H_{46}O_3$ (442.66): C, 78.68; H, 10.48. Found: C, 78.80, 78.75; H, 10.45, 10.67.

Wintersteiner and Moore[11] report: m.p. 141.5–142.5°, α_D −62° Chf, λ^{EtOH} 262.5 mμ (9,500); our sample showed no depression in m.p. when mixed with theirs.

Action of Zinc and Acetic Acid on Cholesteryl Acetate-α,β-oxide.—Refluxing of the mixture for 24 hr. and crystallization from methanol afforded as the chief product a substance that crystallized in needles, m.p. 167–168°, and was identified as cholestane-3β,5α,6β-triol 3,6-diacetate by mixed m.p. determination.

Elimination of Lathosterol by Selenium Dioxide Oxidation.—A solution of 2.5 g. of cholesteryl acetate (through cholesterol dibromide) and 500 mg. of Δ^7-cholestenyl acetate in 700 cc. of ether and 70 cc. of acetic acid was cooled to 0°, treated with 10 cc. of 0.1 *M* selenous acid in acetic acid, and let stand overnight at 0°. The solution containing suspended selenium was diluted with water, extracted with ether, and the washed and dried solution evaporated to a yellow oil containing colloidal selenium. This was dissolved in petroleum ether and adsorbed on a column of alumina introduced on top of a 2-cm. lower layer of precipitated silver for retention of selenium (see paper IV). Elution with petroleum ether gave a series of fractions of cholesteryl acetate, m.p. 111–113°, totalling 2.28 g. (91% recovery); crystallization from methanol gave pure acetate, m.p. 114–115°. Three further elutions with petroleum ether removed no trace of material. The column was then stripped with benzene, which removed 0.50 g. of a yellow oil consisting of a mixture of products derived from the oxidation and dehydrogenation of Δ^7-cholestenyl acetate (see paper IV). The removal of lathosterol was thus practically quantitative.

In one attempt to apply the method, 5 g. of mother liquor cholesterol (B_1) from acetic acid crystallizations of 100 g. of Wilson cholesterol was acetylated, treated with selenium dioxide as above and chromatographed; the petroleum ether eluates all melted in the range 107–112° and benzene then eluted 0.42 g. of yellow oil, corresponding to the presence of 10% of lathosterol in the B_1 fraction. In another experiment, 20 g. of the acetic acid crystallizate A_1 was processed similarly and the petroleum ether eluted material saponified and examined by oxidative analysis, but no new product was detected. Examination of the B_1 fraction (35.5 g.) from crystallization of 600 g. of Wilson cholesterol by the same method was likewise fruitless.

Separation of Cholestanol from Cholesterol and Lathosterol.—A mixture of 4.50 g. of cholesterol (from the dibromide), 250 mg. of cholestanol and 250 mg. of Δ^7-cholestenol in 100 cc. of benzene and 150 cc. of acetic acid was cooled to 0°, treated with a solution of 12.8 g. of dichromate in 50 cc. of acetic acid, and kept at 8–10° for 8 hr. and then at 25° for 15 hr. Processing in the usual way gave 2.70 g. of an acidic fraction (a glass) and 2.73 g. of neutral material. The latter on digestion with 60–90° ligroin gave 70 mg. of white powder melting over 200° (3,6-dione-5α-ol). Extraction of the ligroin mother liquor with Claisen alkali left 0.33 g. of unextracted material that on chromatography and crystallization from methanol of the fractions eluted by 1:1 petroleum ether–benzene gave a total of 184 mg. of cholestanone (74% recovery); the bulk of this melted at 129–130°, mixed m.p. 129.5–130.5°.

Isolation of Ketone 104.—The method of oxidation employed in the first isolation of ketone 104 and in a number of subsequent oxidations of an analytical nature was exactly as described in procedure B (paper II): 20 g. of sterol in 200 cc. each of benzene and acetic acid, treated with 51.2 g. of dichromate in 200 cc. of acetic acid overnight at 9–15° (the temperature is very critical). The procedure of work-up was also the same (ether extraction, digestion with 200 cc. of hexane) down to the point of concentrating the filtrate from cholestane-3,6-dione-5α-ol. The evaporation was carried to a volume of 170 cc. (not 70 cc.) and the Δ^4-cholestene-3,6-dione let crystallize (5–7 g., m.p. 123–124°). The mother liquor was then extracted with 5–6 portions of Claisen alkali and the residue (0.5–0.8 g.) chromatographed. The first few fractions eluted by petroleum ether usually contain small amounts of gums. Cholestanone if present is eluted next and is recognizable from its behavior on crystallization from methanol: it solidifies readily, is less soluble than ketone 104 and separates in small white dots. Ketone 104 comes out in the late fractions eluted by petroleum ether, and the separation from cholestanone is fairly sharp. In the case of the best fractions of the new ketone, the rim of colorless glass left on evaporation turns to a white solid when covered with methanol and when this is dissolved in a small volume of solvent ketone 104 separates only after several hours and then appears in very characteristic long, slender needles, m.p. 123.5–124.5°.

The first sample for analysis was obtained from A_1 cholesterol and was accompanied by only a few mg. of cholestanone, m.p. 129–130°; the yield from 20 g. was 47 mg. Two other analytical samples prepared from A_1 and A_2 likewise melted at 123.5–124.5°; α_D −36.6, −37.6° (*c* 1.07, 1.33); λ^{Chf} 5.81, 8.98, 11.08 μ; saturated to tetranitromethane; unchanged by refluxing overnight with zinc and acetic acid or by refluxing for 2 hr. with 95% ethanol (10 cc.)–36% hydrochloric acid (0.2 cc.).

Anal. Calcd. for $C_{27}H_{44}O_3$ (416.62): C, 77.83; H, 10.65. Found: C, 77.50, 77.69, 77.64, 78.01; H, 10.51, 10.76, 10.76, 10.75.

The yields obtained by this standard procedure seem reproducible and hence the results of the following exploratory oxidations are probably significant, at least in indicating trends. The yield from A_2 cholesterol was 40 mg., or about the same as from A_1, and cholestanone was absent. A_3 gave 107 mg. of ketone 104; B_1 gave 25 mg., and B_2 + B_4 gave only 14 mg. Particularly significant is the observation that on purification of A_2 through the dibromide the yield rose from 40 to 115 mg. From more reliable later results it appeared that the over-all yield from cholesterol *via* the dibromide is about the same as in a direct oxidation.

Comparison of material purified through the dibromide from lots of Wilson Co. cholesterol differing in date of manufacture by about four years suggested that the amount of ketone 104 precursor increase with age. The lot numbers

and yields are as follows: 83879, 85 mg.; 83052, 90 mg.; 74078, 123 mg.; 69768, 139 mg. (the last lot was processed by the improved procedure C described below). The yields of cholestane-3,6-dione-5α-ol in the first three oxidations were 447, 437 and 440 mg. Batches of cholesterol from South American shark-liver oil and skipjack-liver oil after purification through the dibromide gave the following yields: ketone 104, 106 and 110 mg.; 3,6-dione-5α-ol, 338 and 436 mg.

Attempted Fractionations. (a).—A solution of 150 g. of A_3 cholesterol in ether was brominated with 0.8 mole of bromine in acetic acid and left to crystallize very slowly at 25° (111 g.); a second crop (39 g.) was obtained by concentration and addition of 0.2 mole of bromine, and a third (23 g.) by concentration. Standard oxidation after debromination gave ketone 104 as follows (yield per 20 g.): crop 1, 86 mg.; crop 3, 200 mg. Considerable esterification had occurred, since ketone 104 was preceded in the eluates by cholesteryl acetate, 16 and 654 mg.

(b).—A 170-g. batch of cholesteryl acetate dibromide was fractionally crystallized from ether (top fractions) or ether–acetic acid (tail) by the triangulation scheme and separated into 344 fractions. Top and tail fractions when debrominated and saponified gave (per 20 g.) 133 and 48 mg. of ketone 104.

(c).—A 67-g. batch of A_1 cholesterol was converted to the dibromide and the sterol regenerated and rebrominated. After a total of five brominations the material was debrominated, saponified and oxidized; no ketone 104 was found.

(d).—It seemed possible that ketone 104 or a precursor might be found in either the crude Δ^5-cholestene-3-one prepared as usual or in the ethanol mother liquor, but in a preliminary experiment neither fraction on standard oxidation yielded ketone 104. The mother liquor from 158 g. of Δ^5-cholestene-3-one on evaporation gave an oil that when rubbed with petroleum ether gave 9 g. of solid, m.p. 145–150°. This on oxidation and crystallization of the neutral residue from hexane gave 1 g. of crude cholestane-3,6-dione, m.p. 158–161°; recrystallized from hexane, and twice from ethanol, the substance was obtained as short needles, m.p. 170–171°, mixed m.p. 170.5–171.5° (found: C, 80.77; H, 11.12). The hexane mother liquors on chromatography afforded a little Δ^4-cholestene-3,6-dione, m.p. 115–118° (yellow extract with Claisen alkali) and more cholestanedione. The saturated diketone doubtless arose from isomerization of Δ^4-cholestene-6β-ol-3-one.

(e).—Purified cholesterol (93 g., from dibromide) from 74078 was submitted to countercurrent distribution in eight 2-l. separatory funnels in a two-phase system prepared, for each operation, by shaking a mixture of 45 cc. of water with 500 cc. each of benzene, methanol and petroleum ether (30–60°); at the start the sterol was dissolved in the benzene and the other solvents added. Material recovered from the terminal lower layers was largely ether-insoluble (A); the total ether-soluble lower layer material (2 g., m.p. 135–147°) on oxidation gave no ketone 104. Oxidations of 20 g. each of upper layer material gave the following amounts of ketone 104; plates 1, 2: 115 mg.; plates 6, 7, 8: 131 mg.

Materials similar to the hydrophilic tail fraction A were obtained as follows. On digestion of 150 g. of 74078 cholesterol with 1.5 l. of hexane a little white solid remained undissolved. The hot solution was filtered and the solid digested with fresh solvent and collected to give 0.51 g. of material (B). This decomposed at about 160°, was very soluble in methanol or acetic acid, not very soluble in benzene and fairly soluble in water, with frothing. Neither A nor B on oxidation gave any detectable ketone 104. In an earlier experiment chromatography of 10 g. of Wilson cholesterol afforded some 30 fractions totalling 8.3 g. that were indistinguishable in m.p. After exhaustive elution with benzene and then ether, methanol eluted 0.6 g. of yellowish material (C) that was very soluble in methanol or acetone and sparingly soluble in ligroin. After acetylation chromatography afforded a small crop of needles (from methanol), m.p. 158–159°, and a larger fraction, m.p. 119–121°.

Girard Separation of Ketone 104 and Cholestanone.—Although the two ketones left in the residue from Claisen alkali extraction of the neutral fraction from the oxidation of cholesterol can be separated reasonably efficiently by chromatography on a small scale, the process is not adaptable either to large scale preparation of ketone 104 or to analytical determination of the two ketones. The efficiency of effecting separation with Girard's reagent was tested by oxidizing a mixture of 20 g. of purified (dibromide) 69768 cholesterol and 500 mg. of cholestanol and letting the Claisen residue stand in methanol–acetic acid with 0.5 g. of Girard reagent T for 1 hr. The Girard-positive fraction, processed as described in the next section, afforded crude ketonic material that yielded 520 mg. of cholestanone semicarbazone, m.p. 220–224°, corresponding to a recovery of 91%.

Procedure C. Petroleum Ether Phase-Separation.—The convenient Girard separation was incorporated into the following procedure, a second new feature of which is a method of work-up that greatly expedites isolation of ketone 104, cholestanone, and Δ^4-cholestene-3,6-dione. In the example cited the procedure is applied to cholesterol purified through the dibromide, but for the purpose of preparing either ketone 104 or Δ^4-cholestene-3,6-dione in pure form ordinary cholesterol is just as satisfactory.

Oxidation of 20 g. of purified 69768 cholesterol with 10 oxygen equiv. of dichromate at 9–15° was conducted exactly as in procedure B (paper II). Then the brown solution was poured into a separatory funnel, 200 cc. of 30–60° petroleum ether was added, and the mixture shaken and allowed to settle. A dark, chromium-containing lower layer of 240 cc. was drawn off and discarded (this contains the bulk of the acidic products and only a little enedione); the interface with the much lighter colored upper layer is readily observable by suitable illumination from the back. The upper layer was then shaken with 50 cc. of water, and a lower layer of 240 cc. discarded; the washing was repeated and 110 cc. discarded. The now only slightly colored hydrocarbon layer was finally washed with 100 cc. of Claisen alkali and let settle briefly. The lower layer containing the enolate is bright yellow at the bottom, and a heavy brown oil separates below the interface. The liquor and oil were drawn off into a mixture of ice, water and 200 cc. of 36% hydrochloric acid overlayered with ether. A second extract with 100 cc. of Claisen alkali (bright yellow) was run into the same container, which was set aside. The hydrocarbon layer was then washed with seven 100-cc. portions of Claisen alkali and the feebly colored extracts discarded (these contain a little enedione and enolone; see below). The hydrocarbon solution was then evaporated to dryness, evacuated to remove all the benzene, and the residue digested with 40 cc. of methanol and the solution let cool, treated with charcoal, filtered from a little tar and let stand for 1 hr. after addition of 0.5 g. of Girard reagent T and 2 cc. of acetic acid. The solution was diluted, treated with enough alkali to neutralize the acid, extracted with ether and the aqueous solution discarded. The material recovered from the ether (0.5 g.) when taken up in petroleum ether deposited 60 mg. of Δ^4-cholestene-6β-ol-3-one, m.p. 178–180° (needles from hexane, m.p. 191–192°). On chromatography of the mother liquor, 4:1 petroleum ether–benzene eluted material that on crystallization from methanol afforded 139 mg. of needles of pure ketone 104, m.p. 123–124°.

Ether extraction of the acidified yellow Claisen extract gave 8.3 g. of solid that on crystallization from methanol gave in the first crop 5.50 g. (27%) of buff yellow plates of Δ^4-cholestene-3,6-dione, m.p. 124–125°.

In this procedure C, as in B, oxidation is conducted at a temperature (9–15°) optimum for conversion of cholesterol to Δ^4-cholestene-3,6-dione. The more of this material there is present, the more tedious is its removal by extraction with Claisen alkali. It was shown in paper II that at 25° Δ^4-cholestene-3,6-dione is largely oxidized by dichromate to acids, and trials showed that ketone 104 and cholestanone are stable under comparable conditions (they are attacked by chromic anhydride in anhydrous acetic acid). Hence an improvement is to conduct the oxidation for 3–4 hr. at 9–15° and then let it proceed overnight at 25°. After addition of petrolem ether the acids are eliminated in the acetic acid–water–chromate layer that is discarded, and the small amount of enedione remaining is easily removed by brief extraction with Claisen alkali. Thus 50 g. of unpurified 74078 cholesterol yielded 208 mg. of pure ketone 104 in a very brief working period.

CAMBRIDGE, MASS.