phy in toluene–propylene glycol showed trace amounts of II and a large amount of a non-ultraviolet-absorbing product (phosphomolybdic acid spray reagent). Rechromatography of 0.104 g. of the recrystallized solid on 15 g. of Florisil gave, in the 50% ether-in-hexane fractions, 0.033 g., m.p. 151.5–153° (transition at 146°) which was homogeneous, $[\alpha]^{25}D$ +144.9° (dioxane), $[\alpha]^{25}D$ +37.4° (chloroform); λ_{Nujol}^{max} 2.92 (OH), 5.88, 5.92 μ (3- and 20-carbonyl).

Anal. Calcd. for $C_{21}H_{30}O_3$: C, 76.32; H, 9.15. Found: C, 76.44; H, 8.55.

The configuration at 5 has not been assigned. In other respects we assume that the structure derives from II.

Nuclear Magnetic Resonance Spectra.—The nuclear magnetic resonance spectra were determined using a Varian Associate model 4300 V high resolution spectrometer with super stabilizer and spinning sample. The resonance frequencies are reported in c.p.s. at 40 mc. relative to chloroform and were determined *via* the procedure given by Shoolery and Rogers.[5] Vinyl proton areas were measured relative to the area of the quaternary methyl protons. In all cases deuteriochloroform was used as solvent and the reference was chloroform added to the sample in a capillary tube.

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Steroidal Hormone Analogs. V. The Reaction of Cholestane-3-one with Diazomethane[1]

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The reaction of cholestane-3-one with diazomethane afforded A-homocholestane-4-one as the major product. This seven-membered ring ketone was shown to be identical with the ketone obtained from the Tiffeneau ring enlargement of 3-hydroxy-3-aminomethylcholestane. A-Homocholestane-3-one and A-bishomocholestanone were isolated as minor products from the diazomethane ring enlargement reaction. A-Homocholestane-3-one was synthesized by an unambiguous method involving bis-homologation of 2,3-secocholestane-2,3-dioic acid *via* the Arndt–Eistert sequence and pyrolysis of the thorium salt of the resulting diacid. A-Homocholestane-4-one was converted to the 3-hydroxymethylene derivative, thus illustrating the position of substitution in base-catalyzed reactions of this system.

In continuation of our work on the modification of steroidal A-rings[3] we wish to describe methods for the elaboration of A-homosteroids having an oxygen substituent at the 3- or 4-position. The studies, performed on cholestane derivatives, are of such a nature as to be applicable to the preparation of A-homosteroidal hormones.

The one-step ring expansion of a cyclic ketone with diazomethane offers an attractive route to homologous ketones provided the desired product can be separated from unchanged starting material and other products. Under the proper reaction conditions, the predominant product from a six-membered ring ketone will be the seven-membered ring homolog.[4] In the case of an unsymmetrically substituted cyclohexanone (*e.g.*, cholestane-3-one), two seven-membered ring ketones are theoretically possible. However, in similar rearrangement reactions carried out in the D-homosteroid series, it has been shown[5] that a preponderance of one isomer is usually formed. An A-homocholestanone has been synthesized (previously by a Tiffeneau ring-enlargement reaction), but the position taken by the keto group was not determined.[6]

In our work, the first method investigated for the preparation of A-homocholestanones involved the direct ring expansion of cholestane-3-one (I) with diazomethane. The ketone I was treated with a large excess of diazomethane generated *in situ* from N-methylnitrosourea. The product was a mixture from which the seven- and eight-membered ring ketones II–IV were isolated by chromatography. The infrared spectra of the ketones differed in the fingerprint region and this provided a means of telling which fractions collected in the chromatogram represented sufficiently pure material to be combined. A-Homocholestane-3-one (III) exhibits two bands of very weak intensity at 1333 and 1315 cm.$^{-1}$. A-Homocholestane-4-one (II), on the other hand, shows one absorption band at 1333 cm.$^{-1}$. A-Bishomocholestanone (IV) possesses a single band at 1320 cm.$^{-1}$. After chromatography the yields of crude ketones showing these spectral characteristics were II, 40–50%; III, 10%; and IV, 5%. Recrystallization of these compounds to constant melting points gave 27% of II, 0.5–1% of III and 2% of IV.

It was expected that the Tiffeneau ring enlargenent of cholestane-3-one should give as the main ketonic material a substance identical with the major ketone isolated from the diazomethane reaction, because of the similarity of the reaction mechanisms involved. Cholestane-3-one cyanohydrin acetate (V)[6] was reduced with lithium aluminum hydride and the product isolated as the oxazolidine derivative VI. Hydrolysis and deamination of VI with nitrous acid gave a ketone which was purified through its semicarbazone derivative and chromatography. The ketone was shown to be identical with II by a mixed melting point determination and comparison of their infrared spectra. This synthesis also established that compound II from the diazomethane ring enlargement of I is a seven-membered ring ketone.[7]

(1) Abstracted from the thesis submitted by Robert N. Schut to the Massachusetts Institute of Technology, 1958, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(2) Public Health Service Research Fellow of the National Cancer Institute, 1955–1958.

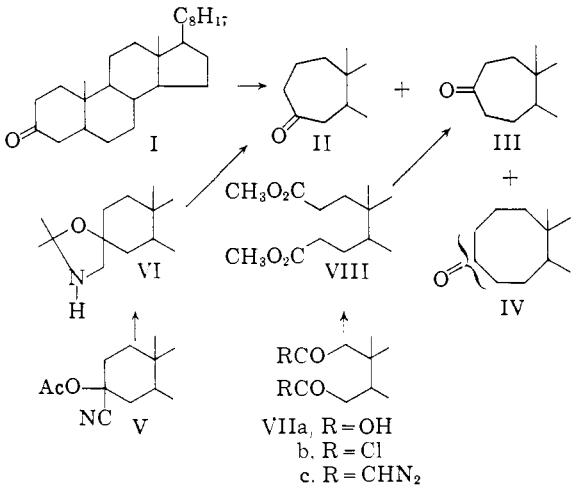
(3) N. A. Nelson and R. N. Schut, THIS JOURNAL, **80**, 6630 (1958).

(4) C. D. Gutsche and H. H. Peter, *ibid.*, **77**, 5871 (1955).

(5) See, for example, N. L. Wendler, D. Taub and H. L. Slates, *ibid.*, **77**, 3559 (1955), and R. O. Clinton, R. G. Christiansen, H. C. Neumann and S. C. Laskowski, *ibid.*, **79**, 6475 (1957).

(6) M. W. Goldberg and H. Kirchensteiner, *Helv. Chim. Acta*, **26**, 288 (1943).

(7) An elemental analysis, within experimental error, does not allow one to distinguish between seven-, eight- or nine-membered ring ketones of this homologous series.



The formulation of ketone IV as an A-bishomocholestanone has not been rigorously established.[7,8] The carbonyl absorption band of the ketone occurs at 1698 cm.$^{-1}$, 5–7 cm.$^{-1}$ lower than the seven-membered ring ketones.

The synthesis of A-homocholestane-3-one by an unambiguous method involved a bis-homologation of 2,3-secocholestane-2,3-dioic acid (VIIa) *via* the Arndt–Eistert sequence followed by ring closure of the resulting diacid. A few problems deserving discussion were encountered along the synthetic route. Treatment of the sodium salt of 2,3-secocholestane-2,3-dioic acid with oxalyl chloride led to the formation of the bis-acid chloride VIIb. The presence of a band at 1755 cm.$^{-1}$ in addition to the expected carbonyl absorption at 1800 cm.$^{-1}$ in the infrared spectrum of the product indicated the presence of some of the corresponding seven-membered ring anhydride. Altering the reaction conditions by adding the sodium salt of VIIa to a large excess of oxalyl chloride failed to arrest the formation of the anhydride.

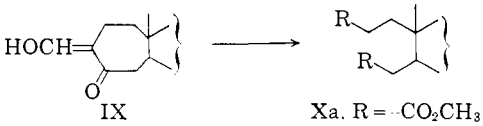
The addition of the crude acid chloride to an excess of ethereal diazomethane yielded impure bis-diazomethyl ketone VIIc. The infrared spectrum of the product showed in addition to the expected strong absorption bands at 2100 and 1635 cm.$^{-1}$,[9] weaker bands at 1800, 1755 (anhydride) and 1735 cm.$^{-1}$. The appearance of the band at 1735 cm.$^{-1}$ may be due to an ester group formed by the action of diazomethane on the anhydride impurity in the starting material. Chromatographic purification of the product using Florisil gave crystalline bis-diazomethyl ketone VIIc in an over-all yield of 43% from the starting dicarboxylic acid VIIa. The infrared spectrum of VIIc exhibited strong bands at 2100, 1635 amd 1360 cm.$^{-1}$ in agreement with the values reported[9] for some aliphatic diazo ketones.

The Wolff rearrangement of VIIc, which was catalyzed by silver benzoate–triethylamine,[10] proceeded smoothly in methanolic solution to give the dimethyl ester VIII. The Dieckmann cyclization of this material under high dilution conditions[11] failed. When the diester was converted to the thorium salt and pyrolyzed, the ketone III was obtained in 35% yield. Chromatographic purification of the ketone gave a crystalline product which was shown by mixed melting point and spectroscopic determinations to be identical with A-homocholestane-3-one obtained from the diazomethane reaction.

It may be concluded, therefore, that the seven-membered ring ketone isolated as the major product in the diazomethane and Tiffeneau ring enlargements of cholestane-3-one is A-homocholestane-4-one (II). Thus in these reactions, 2,3-bond migration occurs predominantly in contrast to the 3,4-bond transposition observed in the Baeyer–Villager oxidation of cholestane-3-one.[12,13]

The base-catalyzed condensation of A-homocholestane-4-one with ethyl formate gave a hydroxymethylene derivative in excellent yield. The product has been formulated as 3-hydroxymethylene-A-homocholestane-4-one (IX) on the basis of the following degradation experiments. Ozonolysis of the hydroxymethylene ketone and decomposition of the ozonide afforded a 40% yield of a dicarboxylic acid, rather than the expected[15] α-diketone. The acid was converted to the dimethyl ester which was shown by a mixed melting point determination and comparison of infrared spectra to be identical with an authentic sample[3] of dimethyl 3,4-seco-A-homocholestanedicarboxylate (Xa).

The oxidative cleavage of the hydroxymethylene ketone IX with alkaline hydrogen peroxide gave a dicarboxylic acid which was converted to an oily dimethyl ester (40% from IX) with diazomethane. The infrared spectrum of the product is identical with the spectrum of an authentic sample of dimethyl 3,4-seco-A-homocholestanedicarboxylate (Xa), and after crystallization of the product there was no depression in melting point on admixture with a sample of authentic Xa. Lithium aluminum hydride reduction of the crude diester produced a diol whose identity with 3,4-seco-A-homocholestane-3,4-diol (Xb)[3] was established by mixed melting point and spectroscopic determinations.



(8) In an analogous case, treatment of *trans*-α-decalone with excess diazomethane has been reported to yield a bishomologated ketone.[4]

(9) P. Yates and B. L. Shapiro with N. Yoda and J. Fugger, THIS JOURNAL, **79**, 5756 (1957).

(10) M. S. Newman and P. F. Beal III, *ibid.*, **72**, 5163 (1950).

(11) F. F. Blicke, J. Azuara, N. J. Doorenbos and E. B. Hotelling, *ibid.*, **75**, 5418 (1953).

(12) V. Burckhardt and T. Reichstein, *Helv. Chim. Acta*, **25**, 1434 (1942).

(13) A marked contrast in migratory aptitudes has also been reported for nitrous acid deamination reactions and peracid rearrangements involving the D-ring of steroids, the former reaction leading mainly to 16,17-bond migration[14] and the latter to nearly exclusive 13,17-bond migration.[5] Although both the peracid oxidation of ketones and the nitrous acid deamination of aminomethylhydroxy compounds involve migration to an electron-deficient center, it is clear that an important mechanistic difference exists in the two types of reactions. Recently R. R. Sauers [THIS JOURNAL, **81**, 925 (1959)] demonstrated the importance of acidity in reversing migratory aptitudes in the peracetic acid oxidation of camphor.

(14) See, however, F. Ramirez and S. Stafiej, *ibid.*, **78** 644 (1956); **77**, 134 (1955).

(15) D. Caunt, W. D. Crow and R. D. Haworth, *J. Chem. Soc.*, 1313 (1951).

The degradation products described above originate from 3-hydroxymethylene-A-homocholestane-4-one. While these compounds have not been obtained in high yield, the fact that they are the only crystalline products which could be isolated suggests strongly that substitution reactions involving enolization of A-homocholestane-4-one occur mainly at C_3.[16] Goldberg and Kirchensteiner[6] tried to determine the position of the carbonyl function of the A-homocholestanone obtained from the Tiffeneau ring enlargement of cholestane-3-one by the formation of the dibenzylidene derivative and subsequent oxidation of this derivative to a known dicarboxylic acid. The experiment failed because the only product which could be isolated was a monobenzylidene derivative. Likewise, bromination of the ketone gave a single crystalline product in 37% yield which was shown to be a monobromo ketone. In accordance with the structural assignment of the hydroxymethylene ketone IX, these compounds can now be considered as 3-substituted derivatives of A-homocholestane-4-one.

Experimental[18]

The Reaction of Diazomethane with Cholestane-3-one (I).—To a solution of 13.4 g. (0.035 mole) of cholestane-3-one[19] in 500 ml. of absolute ether and 850 ml. of absolute methanol was added 28 g. of potassium hydroxide. When all the base had dissolved, the solution was cooled to 0° and 20 g. (0.19 mole) of N-methylnitrosourea was added over a 20-minute period with stirring. Stirring was continued at 0° for an additional 5 hours and then 300 ml. of cold 10% hydrochloric acid was added. The insoluble salts were filtered and washed with ether. The filtrate was concentrated under reduced pressure to remove the organic solvents and the resulting aqueous suspension was extracted with ether. The ether extracts were washed with water, dried and concentrated to give 14.0 g. of a white solid, $\nu_{max}^{CCl_4}$ 1705–1702 cm.$^{-1}$.

The crude ketone was chromatographed on 600 g. of Merck acid-washed alumina (Brockmann activity 2). Elution with hexane–ether (4:1) gave as the main fraction, 8.50 g. of a white solid, m.p. 77–82°. The material was recrystallized from methanol to give 5.77 g. (41%) of **A-homocholestane-4-one (II)**, m.p. 85.5–87.5°, $\nu_{max}^{CCl_4}$ 1704 cm.$^{-1}$ (s, CO stretching) and a characteristic single weak band at 1333 cm.$^{-1}$. One more recrystallization from methanol gave 3.79 g. (27%) of small plates, m.p. 87.0–88.0°. This ketone showed no melting point depression when mixed with a sample of the ketone obtained from the Tiffeneau ring enlargement (see below).

Anal. Calcd. for $C_{28}H_{48}O$: C, 83.93; H, 12.08. Found: C, 83.70; H, 11.93.

A-Homocholestane-4-one 2,4-dinitrophenylhydrazone, after chromatography on Merck acid-washed alumina (eluted with hexane–ether 9:1), was recrystallized from ethanol–chloroform; m.p. 193.5–194.5°.

Anal. Calcd. for $C_{34}H_{52}O_4N_4$: C, 70.31; H, 9.02; N, 9.65. Found: C, 70.24; H, 9.01; N, 9.73.

In another run 16.0 g. of the ketonic mixture was chromatographed on 1600 g. of Merck acid-washed alumina (Brockmann activity 1). The products were eluted with one-hundred and forty 100-ml. portions of hexane–ether mixtures of increasing polarity. Those fractions having the proper infrared spectral characteristics (eluted with hexane–ether mixtures containing 20–65% ether) were combined yielding: (1) 0.91 g. (5%) of white solid, m.p. 90–100°, $\nu_{max}^{CCl_4}$ 1698 cm.$^{-1}$ and a single weak band at 1320 cm.$^{-1}$; (2) 1.90 g. (12%) of white solid, m.p. 70–75°, $\nu_{max}^{CCl_4}$ 1703 cm.$^{-1}$ and two weak bands at 1333 and 1315 cm.$^{-1}$; (3) 6.34 g. (40%) of II, m.p. 82–84°.

Fraction 1 on recrystallization from methanol gave 0.43 g. (2%) of white needles, m.p. 110–111°. Further recrystallization from methanol gave an analytical sample of A-bishomocholestanone (IV), m.p. 111.5–112.0°.

Anal. Calcd. for $C_{29}H_{50}O$: C, 83.99; H, 12.15. Found: C, 84.25; H, 12.26.

Fraction 2 was recrystallized several times from methanol to give 0.09 g. (0.5%) of III in the form of fine white needles, m.p. 84.5–85.5°. A mixed melting point determination with A-homocholestane-3-one obtained from the thorium salt pyrolysis (see below) showed no depression. Admixture of this material with a sample of A-homocholestane-4-one (m.p. 87–88°) depressed the melting point (80.5–84°).

3-(5′-Spiro-2′,2′-dimethyloxazolidinyl)-cholestane (VI).[20]—A solution of 8.50 g. of cholestane-3-one cyanohydrin acetate[6] (V) in 100 ml. of anhydrous ether and 100 ml. of anhydrous benzene was added with stirring to a slurry of 6 g. of lithium aluminum hydride in 250 ml. of anhydrous ether over a period of 45 minutes. The mixture was stirred at room temperature for 30 minutes and then heated under reflux for 30 minutes. The excess hydride was decomposed by the cautious addition of water and the organic solvents were distilled *in vacuo*. The resulting suspension was basified with 200 ml. of 10% sodium hydroxide solution and filtered. The white solid obtained was extracted continuously (Soxhlet apparatus) with acetone for 24 hours and the resulting solution was concentrated to give 5.84 g. (68%) of the oxazolidinyl derivative VI, m.p. 145–146°. An analytical sample recrystallized twice from acetone and then sublimed (180–200°, 0.1 mm.) melted at 145–146°.

Anal. Calcd. for $C_{31}H_{55}NO$: C, 81.33; H, 12.11; N, 3.06. Found: C, 81.43; H, 12.16; N, 3.04.

A-Homocholestane-4-one (II).—To a stirred solution of 4.58 g. of 3-(5′-spiro-2′,2′-dimethyloxazolidinyl)-cholestane in 800 ml. of aqueous 10% acetic acid maintained at 0–5° was added a solution of 2 g. of sodium nitrite in 100 ml. of water over a 1-hour period. Stirring was continued at 0–5° for 3 hours and the mixture was allowed to stand overnight. The reaction mixture was neutralized with 10% sodium hydroxide solution and the resulting white suspension was extracted with ether. The ether extracts were washed with water, dried and concentrated to give a semi-solid residue which was converted to the semicarbazone. The crude product was recrystallized from ethanol in the form of a white powder, m.p. 239–241° dec. (lit.[6] 239–240°). A solution of 50 ml. of hydrochloric acid in 450 ml. of ethanol was added to the semicarbazone and the mixture was heated under reflux for 1 hour. The clear solution was diluted with 250 ml. of water and the resulting precipitate was collected and washed with a little cold methanol. Recrystallization of this material from methanol afforded 1.14 g. (28%) of white crystals, m.p. 80–86°. Chromatographic purification of the ketone on 80 g. of Merck acid-washed alumina with hexane–ether (9:1) as the eluent provided after recrystallization from methanol 0.69 g. of A-homocholestane-4-one as white needles, m.p. 87.5–88.0° (lit.[6] 85–87°).

A-Bishomo-3,4-secocholestane-3,4-dioic Acid Dimethyl Ester (VIII).—A 10.1-g. sample of 2,3-secocholestane-2,3-dioic acid (VIIa)[21] was neutralized with 0.87 *M* methanolic sodium methoxide solution and the methanol was then re-

(16) Directional specificity in substitution reactions of other steroidal ketone systems is of course well known; for example, monosubstitution reactions of cholestane-3-one (I) yield chiefly the 2-substituted derivatives. The explanations that have been advanced[17] for the directional specificity observed in the reactions of I may apply equally well for the reactions of A-homocholestane-4-one (II). However, in the latter case steric factors should also be considered. From an examination of molecular models of II it is apparent that in some conformations of the seven-membered A-ring, the 4a-position is sterically hindered toward the approach of a reagent whereas the 3-position appears to be relatively unhindered in all conformations.

(17) R. B. Turner, W. R. Meador and R. E. Winkler, THIS JOURNAL, **79**, 4122 (1957); E. J. Corey and R. A. Sneen, *ibid.*, **77**, 2505 (1955).

(18) Melting points are uncorrected. The infrared spectra were determined with a Baird or Perkin–Elmer (model 21) spectrophotometer fitted with a sodium chloride prism. In reporting infrared spectra, (s) denotes strong, (m) medium and (w) weak absorption. The microanalyses were performed by Dr. S. M. Nagy and associates.

(19) L. F. Fieser and X. A. Dominguez, THIS JOURNAL, **75**, 1704 (1953).

(20) *Cf.*, H. Heusser, P. Th. Herzig, A. Furst and Pl. A. Plattner, *Helv. Chim. Acta*, **33**, 1093 (1950).

(21) B. H. Brown, I. M. Heilbron and E. R. H. Jones, *J. Chem. Soc.*, 1482 (1940).

moved *in vacuo*. The pulverized salt was dried (100°, 1.5 mm., 12 hours) and added in small portions over a 30-minute period to a cold solution of 15 g. of redistilled oxalyl chloride in 50 ml. of anhydrous benzene and 1 ml. of pyridine (nitrogen atmosphere). The mixture was stirred at 0° for an additional 15 minutes before removing the organic solvents *in vacuo*. Benzene was added and removed *in vacuo* and the residue was dried at 0.1 mm. for 12 hours. An infrared spectrum (CCl_4) of the crude bisacid chloride showed bands at 1800 (s, acid chloride CO and possibly some anhydride CO) and 1755 cm.$^{-1}$ (m, anhydride CO). Anhydrous benzene (100 ml.) was added to the residue and the resulting mixture was filtered to remove insoluble salts. The filtrate was added with stirring over a 1-hour period to 360 ml. of a cold (−15°) ethereal solution of 2.8 *M* diazomethane which had been distilled and dried at 0° over potassium hydroxide for 1 hour and over sodium wire for 2 hours.[22] The yellow solution was stirred for 2 hours at room temperature before the solvents were removed *in vacuo* to yield 10.0 g. of crude product. Chromatographic purification of this material on 140 g. of Florisil (100–200 mesh) using hexane–acetone (9:1) eluent gave 4.77 g. (43%) of the bisdiazomethyl ketone VIIc as a light yellow solid, m.p. 114–116° dec., $\nu_{max}^{CCl_4}$ 3100(w), 2100 (s, $-CHN_2$ grouping), 1635 (s, $-COCHN_2$ grouping) and 1360 cm.$^{-1}$(s).

The Newman–Beal modification[10] of the Wolff rearrangement was employed in the following procedure. To a stirred solution of 4.77 g. of the bisdiazomethyl ketone VIIc in 100 ml. of anhydrous ether and 100 ml. of absolute methanol was added a solution of 1.5 g. of silver benzoate in 25 ml. of redistilled triethylamine. A 2-ml. portion of the silver benzoate solution was added rapidly at first; a black precipitate of silver formed almost immediately. The remainder of the silver benzoate solution was added over a 1-hour period. The mixture was stirred at room temperature for 1 hour and then heated under reflux for 15 minutes. The mixture was filtered and the organic solvents removed *in vacuo*. The dark residue was taken up in ether and filtered through a 1-cm. layer of acid-washed alumina. After washing the solids with 200 ml. of ether and concentrating the filtrate there remained 4.47 g. of a light yellow oil which solidified on standing overnight. Chromatographic purification of this material on Merck acid-washed alumina (activity 2) with hexane–ether (9:1) eluent gave 3.28 g. (67%) of the diester VIII, m.p. 108–109°. The analytical sample of A-bishomo-3,4-secocholestane-3,4-dioic acid dimethyl ester crystallized from methanol as white needles, m.p. 109.5–110°; $\nu_{max}^{CCl_4}$ 1737 cm.$^{-1}$ (s, ester CO).

Anal. Calcd. for $C_{31}H_{54}O_4$: C, 75.87; H, 11.09. Found: C, 75.67; H, 11.08.

A-Homocholestane-3-one (III).—A-Bishomo-3,4-secocholestane-3,4-dioic acid dimethyl ester (2.65-g.) was saponified by refluxing it for 1.5 hours in a mixture of 50 ml. of methanol and 50 ml. of 5% sodium hydroxide solution. The methanol was removed by distillation and the aqueous residue was washed with ether and neutralized (phenolphthalein end-point) with hydrochloric acid before being warmed to 50° and added to a hot solution of 5 g. of thorium nitrate in 100 ml. of water. After heating the mixture for 10 minutes, the thorium salt was collected on a filter, washed with hot water, pulverized and dried (60°, 2 mm., 12 hours). The thorium salt (3.07 g.) was mixed with an equal weight of electrolytic iron powder and the mixture placed in a sublimation apparatus which was evacuated to 0.001 mm. and inserted in a Wood metal-bath preheated to 250°. The pyrolysis took place at 330–380° over a 2-hour period. The sublimate was dissolved in ether and the solution was filtered and concentrated to give 0.76 g. of a solid. Chromatographic purification of this material on 40 g. of Merck acid-washed alumina (activity 2) using hexane–ether (9:1) eluent gave 0.60 g. (28%) of A-homocholestane-3-one, m.p. 81–83°. The analytical sample of III crystallized from methanol as fine needles, m.p. 85.0–85.5°, $\nu_{max}^{CCl_4}$ 1703 cm.$^{-1}$ (s, CO stretching) and two weak bands at 1333 and 1315 cm.$^{-1}$ characteristic of this substance. The mixed melting point with A-homocholestane-4-one (II, m.p. 87–88°) was depressed (81.5–84°).

Anal. Calcd. for $C_{28}H_{48}O$: C, 83.93; H, 12.08. Found: C, 83.96; H, 12.30.

The 2,4-dinitrophenylhydrazone of III was obtained as fine yellow needles after chromatography on acid-washed alumina and recrystallization from ethanol–chloroform, m.p. 196.5–197°. Admixture of this material with the 2,4-dinitrophenylhydrazone of A-homocholestane-4-one (m.p. 193.5–194.5°) depressed the melting point (188–191°).

Anal. Calcd. for $C_{34}H_{52}O_4N_4$: C, 70.31; H, 9.02; N, 9.65. Found: C, 69.95; H, 8.97; N, 9.61.

3-Hydroxymethylene-A-homocholestane-4-one (IX).—To a suspension of 2.2 g. of freshly prepared sodium methoxide and 15 ml. of reagent-grade benzene under nitrogen was added 5 ml. of ethyl formate (distilled from phosphorus pentoxide). A solution of 1.01 g. of A-homocholestane-4-one in 20 ml. of benzene was added and the mixture was stirred overnight. Water and ether were added followed by 40 ml. of cold 10% hydrochloric acid. The organic layer was separated, washed with distilled water, dried and concentrated to give 1.10 g. of crude product, m.p. 95–107°, $\nu_{max}^{CCl_4}$ 1640 (s, chelated CO) and 1585 cm.$^{-1}$ (m, conj. C=C). The material produced a red-violet color with alcoholic ferric chloride solution. The analytical sample was crystallized from methanol, m.p. 100.5–103°.

Anal. Calcd. for $C_{29}H_{48}O_2$: C, 81.25; H, 11.29. Found: C, 81.06; H, 11.21.

Ozonolysis of 3-Hydroxymethylene-A-homocholestane-4-one (IX).—The general procedure of Caunt, Crow and Haworth[15] was followed. A solution of 0.45 g. of the crude hydroxymethylene ketone IX in 50 ml. of ethyl acetate was cooled to −70° and a stream of ozone–oxygen was bubbled through the solution for 10 minutes. The light blue solution was hydrogenated at −20 to 0° using 1 g. of 10% palladium-on-charcoal catalyst. The mixture was filtered and the filtrate concentrated to give 0.47 g. of a light yellow oil which gave a negative test with ferric chloride solution.

An ethereal solution of the crude product was extracted with base. Acidification of the basic extract gave 191 mg. (40%) of a white solid, m.p. 200–220° dec., ν_{max}^{KBr} 3400–2600 (carboxyl OH), 1705 (s, carboxyl CO) and 930 cm.$^{-1}$ (w, carboxyl dimer). Two recrystallizations of this material from acetic acid–water gave 69 mg. of material with m.p. 224–228° dec. Treatment of this acid with diazomethane and chromatographic purification of the resulting material gave 51 mg. of an oily ester which on crystallization from methanol yielded A-homo-3,4-secocholestane-3,4-dioic acid dimethyl ester (Xa), m.p. 58–59° (lit.[3] 56–57°); the mixed melting point of this material with an authentic sample was not depressed.

Chromatographic purification of the neutral material remaining after the basic extraction of the decomposed ozonide was unsuccessful.

Oxidative Cleavage of 3-Hydroxymethylene-A-homocholestane-4-one (IX).—The general procedure of Ruzicka, Rey and Muhr[23] was utilized. To a solution of 299 mg. of crude hydroxymethylene-A-homocholestane-4-one (IX) in 10 ml. of ethanol was added 10 ml. of 15% sodium hydroxide solution followed by 4 ml. of 30% hydrogen peroxide solution. The mixture was warmed for 20 minutes and to the resulting clear solution were added 20 ml. of 10% sodium hydroxide solution and 3 ml. of 30% hydrogen peroxide. The solution was heated under reflux for 1 hour and then washed with ether. Acidification of the aqueous phase with concentrated hydrochloric acid yielded 218 mg. (70%) of crude A-homo-3,4-secocholestane-3,4-dioic acid, m.p. 200–212° dec. Treatment of the diacid with diazomethane gave after chromatographic purification 141 mg. (62%) of oily dimethyl ester, $\nu_{max}^{CCl_4}$ 1735 cm.$^{-1}$ (s, ester CO), which crystallized on being seeded with a crystal of A-homo-3,4-secocholestane-3,4-dioic acid dimethyl ester (Xa). Recrystallization of the product from methanol gave 78 mg. (35%) of Xa, m.p. 49–54°, raised to 55–57° after two recrystallizations. The mixed melting point of this material with an authentic sample[3] of Xa was not depressed.

The mother liquors from the recrystallizations of the dimethyl ester described above were concentrated to give 76 mg. of an oil whose infrared spectrum was identical with that of authentic Xa. A 70-mg. sample of this oil was reduced with an excess of ethereal lithium aluminum hydride. Isolation of the product in the usual way[3] gave 60 mg. of a

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semi-solid which on crystallization from pentane yielded 29 mg. (47%) of material with m.p. 145–150°. Two recrystallizations of this material from benzene–petroleum ether gave 7 mg. of fine needles, m.p. 158–159°, undepressed by admixture with an authentic specimen[3] of A-homo-3,4-seco-cholestane-3,4-diol (Xb).

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[CONTRIBUTION FROM THE BIOLOGICAL AND MEDICAL SCIENCES DIVISION, U. S. NAVAL RADIOLOGICAL DEFENSE LABORATORY]

A Modified Calcium Phosphate for Column Chromatography of Polynucleotides and Proteins[1]

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Specimens of calcium phosphate, prepared at different *p*H values and subsequently modified by heat or alkali, have been investigated as possible absorbents for the column chromatography of polynucleotides and proteins. One such specimen, precipitated at *p*H 6.7 and boiled with saturated calcium hydroxide has the desired characteristics of ease and reproducibility of preparation, high flow rate, stability, capacity and degree of resolution. It has been used to fractionate deoxyribonucleic acid and polyadenylic acid, to separate the latter from the former, and to separate albumin from the nucleic acid of a 10 to 1 mixture of the two by gradient elution with neutral phosphate buffers.

It is the purpose of this communication to describe the development, preparation, certain chemical and physical properties and chromatographic characteristics of a new calcium phosphate preparation suitable for column chromatographic separation of DNA[2] from certain other macromolecules such as proteins and lower molecular weight polynucleotides. Notable progress recently has been made by a number of workers in the development of adsorbents for column chromatography of proteins,[3–5] of DNA[6–12] and of RNA.[13] For the most part these methods were applied to the resolution of, and demonstration of heterogeneity in, a single previously purified chemical species, *i.e.*, protein, DNA or RNA. Previous reports from this Laboratory[14,15] indicated that DNA could be separated, in mixtures, from the products of minimal enzymatic digestion of DNA (polynucleotides) by elution with a phosphate buffer gradient from calcium phosphate columns, and that by a similar method it could be demonstrated that certain polynucleotides from DNA were liberated in mouse spleen tissue following whole body X-irradiation.[16]

In the present report a systematic study has been made of the effect of *p*H of precipitants, and of heating with alkalies, in the preparation of calcium phosphate for column chromatography. Variations in the resulting calcium phosphate preparations were followed by the criteria of (a) flow rates through standard packed columns, (b) X-ray diffraction powder patterns, (c) chemical analyses (molar calcium/phosphorus ratios) and (d) chromatographic behavior with DNA. These studies have led to a calcium phosphate preparation suitable for the column chromatography of DNA, bovine plasma albumin, polyadenylic acid, RNA,[17] and certain mixtures thereof. In addition, a simplified method of preparing a calcium phosphate with properties similar to those of the semi-hydroxylapatite of Tiselius and co-workers[3] will be described.

Experimental

Calcium Phosphate Preparations.—A series of 0.5 *M* sodium phosphate buffers ranging in *p*H from 6.8 to 7.8 by intervals of 0.2 unit was prepared. Calcium phosphate was precipitated by admixing 100 ml. of 0.5 *M* calcium chloride solution (1 drop per second) with 120 ml. of each buffer under mechanical stirring at room temperature. In this manner phosphate ions were continuously in over-all excess during the entire precipitation process. Each of the resulting suspensions was stirred for one additional hour and then allowed to settle. The *p*H of each supernatant solution was

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(2) The following abbreviations have been used: DNA, deoxyribonucleic acids; RNA, ribonucleic acids; RNAse, ribonuclease. For purposes of this report, calcium phosphate is abbreviated, CP. CP, followed by arabic numerals represents calcium phosphate precipitated by the manner described in the text from sodium phosphate buffer initially of *p*H indicated by the arabic numerals. Thus, CP 6.7 represents a calcium phosphate specimen precipitated from sodium phosphate buffer initially at *p*H 6.7. CPA represents calcium phosphate prepared as described by boiling CP 6.7 in a solution of ammonium hydroxide. CPM represents calcium phosphate prepared by boiling CP 6.7 with calcium hydroxide solution. CPS represents a prepared standard of pure anhydrous secondary calcium orthophosphate while CPHA represents a prepared standard of pure hydroxylapatite. CPT represents a sample of calcium phosphate prepared according to the method of Tiselius.[3]

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