3β-Chloro-9(11)-cholenic acid (**32**), prepared by hydrolysis of its methyl ester, crystallized from ethanol, m.p. 150–153°, $[\alpha]_D$ +33.4°. *Anal.* Calcd. for $C_{24}H_{37}O_2Cl$ (393.00): C, 73.34; H, 9.49. Found: C, 73.4; H, 9.8.

Methyl 3β-chlorocholanate (**33**) by pyridinium chloride method on methyl lithocholate tosylate (20), in 80% yield of crude product melting about 80°. After recrystallization from methanol, well-shaped, elongated prisms of m.p. 86–87.5° were obtained, $[\alpha]_D$ +17°. *Anal.* Calcd. for $C_{25}H_{41}O_2Cl$ (409.03): C, 73.41; H, 10.1. Found: H, 73.1; H, 9.8.

3β-Chlorocholanic acid (**34**), prepared by hydrolysis of the methyl ester 33, separated in long needles, from acetone, m.p. 186–190°, $[\alpha]_D$ +14.3°. *Anal.* Calcd. for $C_{24}H_{39}O_2Cl$ (395.01): C, 72.97; H, 9.95; Cl, 8.98. Found: C, 73.22; H, 9.91; Cl, 9.07.

Methyl 3β-chloro-12α-hydroxycholanate (**35**), by pyridinium chloride on the tosylate 25, crystallized out of methanol as transparent, dense prisms, m.p. 141.5–143°, $[\alpha]_D$ +29.6°*. *Anal.* Calcd. for $C_{25}H_{41}O_3Cl$ (425.04): C, 70.64; H, 9.72; Cl, 8.34. Found: C, 70.81; H, 9.85; Cl, 8.28.

Ethyl 3β-chloro-12α-hydroxycholanate (**36**) by ester interchange on 35, by refluxing for 3.5 hr. with hydrochloric acid in ethanol, 86% yield of fine needles, m.p. 116.5–118.8°. Analytical sample out of ligroin (35–60°)–benzene (20:1), m.p. 117.5–118.8°, $[\alpha]_D$ +28.6°*. *Anal.* Calcd. for $C_{26}H_{43}O_3Cl$ (439.06): C, 71.12; H, 9.87; Cl, 8.08. Found: C, 71.05; H, 9.73; Cl, 7.96.

Methyl 3β-chloro-5-bisnorcholenate (**37**) by the action of thionyl chloride on the 3β-hydroxy compound, m.p. 130–133°, out of methanol in 79% yield, $[\alpha]_D$ −48°. *Anal.* Calcd. for $C_{23}H_{35}O_2Cl$ (378.97): C, 72.89; H, 9.31; Cl, 9.36. Found: C, 72.96; H, 9.01; Cl, 9.44.

Ethyl 3β-chloro-5-bisnorcholenate (**38**) was prepared in a room temperature reaction of thionyl chloride on the hydroxy ester 7 to give a crystalline product from ethanol, m.p. 120–123°, $[\alpha]_D$ −47.3°. *Anal.* Calcd. for $C_{24}H_{37}O_2Cl$ (393.00): C, 73.34; H, 9.49; Cl, 9.02. Found: C, 73.5; H, 9.5; Cl, 8.9.

OTHER COMPOUNDS.—**Ethyl 12α-benzoxycholanate** (**39**) by reaction of benzoyl chloride in pyridine on the 12-hydroxy compound 4, 4 hr. reflux. The oil, after processing and chromatography, did not crystallize; distilled at 0.1 mm., $[\alpha]_D$ +51°. *Anal.* Calcd. for $C_{33}H_{48}O_4$ (508.7): C, 77.91; H, 9.51. Found: C, 78.0; H, 9.6.

Ethyl 3α-benzoxy-12α-hydroxycholanate (**40**) was prepared by benzoylation in pyridine at room temperature; 75% of product, m.p. 103–114°. Recrystallization from methanol, m.p. 118.8–120.7°, $[\alpha]_D$ +51.9°*. *Anal.* Calcd. for $C_{33}H_{48}O_5$ (524.68): C, 75.53; H, 9.22. Found: C, 75.80; H, 9.28.

Ethyl 3α,12α-dibenzoxycholanate (**41**) from ethyl desoxycholate with benzoyl chloride and pyridine by 3 hr. reflux, crystalline product from ethanol, m.p. 139.2–141.4°, $[\alpha]_D$ +89.7°*. *Anal.* Calcd. for $C_{40}H_{52}O_6$ (628.80): C, 76.40; H, 8.33. Found: C, 76.43; H, 8.59.

Ethyl 3-ketocholanate (**42**)[22] by esterification of 3-ketocholanic acid,[23] crystallized out of ethanol–water as dense prisms, m.p. 94.5–95.5°, $[\alpha]_D$ +28.9°. *Anal.* Calcd. for $C_{26}H_{42}O_3$ (402.60): C, 77.56; H, 10.52. Found: C, 77.52; H, 10.70.

Methyl 3-keto-9(11)-cholenate (**43**)[21] was prepared from methyl 3α-hydroxy-9(11)-cholenate by the chromium trioxide–pyridine method[24] to give an 82% yield of colorless crystals, m.p. 117–120°, $[\alpha]_D$ +31.3° (lit. m.p. 117–119°, $[\alpha]_D$ +35°).

Methyl 3-keto-11-cholenate (**44**)[25] from chromium trioxide–pyridine oxidation of the corresponding 3α-hydroxy compound, out of isopropyl ether–acetone (9:1) giving transparent flat plates, m.p. 125–127° (lit. m.p. 125.5–126°), $[\alpha]_D$ +37.3°. *Anal.* Calcd. for $C_{25}H_{38}O_3$ (386.55): C, 77.67; H, 9.91. Found: C, 77.4; H, 10.2.

(22) This compound is mentioned among other carbonyl compounds [H. Reich, K. F. Crane and S. J. Sanfilippo, *J. Org. Chem.*, **18**, 822 (1953)] without indication of its source or properties.

(23) H. Wieland and P. Weyland, *Z. physiol. Chem.*, **110**, 123 (1920).

(24) G. I. Poos, G. E. Arth, R. E. Beyler and L. H. Sarett, THIS JOURNAL, **75**, 422 (1953).

(25) V. Burckhardt and T. Reichstein, *Helv. Chim. Acta*, **25**, 821 (1942).

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[CONTRIBUTION FROM THE DIVISION OF CHEMISTRY, AND THE DIVISION OF PATHOLOGY AND MICROBIOLOGY, MEDICAL UNITS, UNIVERSITY OF TENNESSEE]

Seroflocculating Steroids. IV.[1] Unsaturated Bile Acid Esters[2]

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RECEIVED NOVEMBER 20, 1956

For study of their seroflocculating activity, methyl and ethyl 3,11-choladienate, methyl 3,9(11)-choladienate, methyl and ethyl 3-cholenate, methyl and ethyl 12α-hydroxy-3-cholenate were prepared by elimination of suitably constituted 3α-tosylates. Each elimination reaction appears to give a single (or highly predominant) olefinic product. The assignment of the Δ^3-structure to these compounds is based on the identity of our methyl cholenate with the product prepared previously by Fieser and Ettore and proved by them to be methyl 3-cholenate.

Our first attempts to prepare a pure ethyl choladienate for seroflocculation studies were by pyrolysis of diacyl derivatives of ethyl desoxycholate by the methods used by Wieland[4] and subsequently by Reichstein.[5] Solid products were obtained readily enough, but isolation of pure material by crystallization, or even by chromatography and crystallization, was tedious and gave low yield. For example, with ethyl 3α,12α-dibenzoxycholanate, the pyrolysis product yielded a minute amount of pure material only after chromatography and eight crystallizations. The diacyl methyl esters were no more amenable than the ethyl compounds.

The difficulty of isolation of pure product induced us to try an elimination reaction on a tosylate. Dehydrotosylation in a pyridine base which presumably takes place through a heterolytic mechanism might be expected to yield a different and possibly more tractable mixture than that resulting from pyrolysis. This method of producing unsaturation in steroids has been applied frequently,[6] but the

(1) Paper III of this series, THIS JOURNAL, **79**, 2164 (1957).

(2) This investigation was supported in part by grants (CS-9053 and C-2249) from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

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(4) H. Wieland and W. Kapitel, *Z. physiol. Chem.*, **212**, 269 (1932).

(5) A. Lardon, P. Grandjean, J. Press, H. Reich and T. Reichstein, *Helv. Chim. Acta*, **25**, 1444 (1942).

(6) J. von Euw and T. Reichstein, *ibid.*, **29**, 654 (1946).

composition of the olefinic products obtained in the elimination of cholestanyl tosylates has been studied only recently.[7] And no similar information is available on the A-ring unsaturated products resulting from the 3-tosylates of the coprostane (A/B *cis*) series.

To limit the number of derived products, an ethyl desoxycholate derivative with the preformed 11-unsaturated group, ethyl 3α-hydroxy-11-cholenate[8] was chosen as the starting material. By reflux with tosyl chloride in pyridine solution, a nicely crystalline product was obtained, which, however, proved to be a difficultly separable mixture of diene and 3β-chloro compound, as described in paper II.[9]

When the tosylate 24[10] was first prepared and refluxed in 2,6-lutidine, a solid dienic product was obtained readily from the reaction mixture, but first efforts at purification by fractional crystallization seemed to indicate that, as with the pyrolysis products, the mixture yielded to separation reluctantly. However, by simply passing the material through an alumina column in ligroin, the persistent contaminant was removed and an excellent yield of homogeneous product could be achieved after one or two crystallizations.[10a] The impurity is a minor component that remains adsorbed on the alumina and apparently is not an isomeric diene.

Methyl 3,11-choladienate, methyl 3,9(11)-choladienate, methyl and ethyl 3-cholenate and methyl and ethyl 12α-hydroxy-3-cholenate were all prepared from the respective 3-tosylates in lutidine and purified in analogous manner satisfactorily. Methyl 12α-hydroxy-3-cholenate was obtained in pyridine in only 32% as compared with a 98% yield when lutidine was used. The improved yield when lutidine is used as dehydrotosylation medium in place of pyridine may be attributed partly to the increase in temperature of reaction (pyridine, b.p. 116°; 2,6-lutidine, 143°) but may also possibly be due to the greater steric strain in the transition state required for the formation of the salt when lutidine is the base, as suggested in a similar previous study by King.[11] The replacement reaction competing with elimination would result in the lutidinium (or pyridinium) salt.

Assignment of the Δ^3-Structure.—Fieser and Ettore[12] prepared methyl 3-cholenate by the zinc treatment of the methyl 3-hydroxy-4-bromocholanates, and Yamasaki, Rosnati, Fieser and Fieser[13] showed that it is identical with the methyl ester of an unsaturated acid (originally given the assignment of 2-cholenic acid by Wieland)[14] obtained by pyrolysis of lithocholic acid. They further confirmed the 3-unsaturation by conversion of the methyl cholenate to a 3,4-diol with osmium tetroxide and oxidation of this diol to lithobilianic acid.

Our methyl cholenate obtained from the dehydrotosylation reaction has been compared with the Fieser compound,[13] and the identity of the two preparations was established by comparisons of melting points, optical rotations and infrared spectra and by a mixed melting point determination. Moreover, our compound was converted to the ethyl ester which in turn was shown to be identical with authentic ethyl 3-cholenate provided by Professor Fieser.[13]

The Δ^3-compounds appear to be the sole olefin products of the dehydrotosylation reaction; no Δ^2-isomers were isolated. Wieland[14] reported a 9:1 ratio of isomers in the pyrolysis of lithocholic acid, and in fact his isolation and characterization of the minor component as 3-cholenic acid (now 2-cholenic acid) were extremely tentative. Therefore, the Δ^3-isomer appears to be the sole or highly predominant olefinic product[15] of both the pyrolysis and dehydrotosylation reactions. This is in interesting contrast to the situation of the cholestanyl derivatives; pyrolysis of the 3β-benzoates[16] gave both Δ^2- and Δ^3-cholestene, and elimination of 3α- and 3β-tosylates both afforded a Δ^2- and Δ^3-cholestene mixture in 1:1 ratio.

Consequently, the Δ^3-structure has been assigned to the other compounds made through the dehydrotosylation reaction: ethyl 3-cholenate, methyl 3,9(11)-choladienate, methyl and ethyl 3,11-choladienate, methyl and ethyl 12α-hydroxy-3-cholenate. Methyl 3,11-choladienate, also prepared by the pyrolysis of methyl desoxycholate dibenzoate and brought to satisfactory purity after fourteen recrystallizations, is probably identical with the material reported (m.p. 86°) obtained by Wieland and Kapitel[4] by a similar pyrolysis after "repeated" crystallizations and is most likely the methyl ester of choladienic acid-A. Choladienic acid-A was tentatively considered to be the $\Delta^{2,11}$-compound by Wieland, but Fieser[13] has recently proposed revision to the $\Delta^{3,11}$-structure. Reichstein and his coworkers[5] obtained as a side-product in a pyrolysis reaction of methyl 3α-acetoxy-12α-benzoxycholanate a 76° melting product which they regarded as a mixture of the methyl 2,11- and 3,11-choladienates. The unsaturated compound made by Barnett and Reichstein[17] by dehydrotosylation in pyridine is undoubtedly identical with our methyl 12α-hydroxy-3-cholenate. No supporting evidence or comment was given in the designation of the Δ^3-structure of their product; the preparation appar-

(7) H. R. Nace, THIS JOURNAL, **74**, 5937 (1952).

(8) We are indebted to Dr. K. Pfister and Merck and Co., Inc., for a generous supply of 3α-hydroxy-11-cholenic acid.

(9) F. C. Chang, *et al.*, THIS JOURNAL, **79**, 2161 (1957).

(10) Numbered according to the consecutive order in which the compounds are described in the Experimental sections of this paper IV and the accompanying paper III of this series.

(10a) ADDED IN PROOF.—S. Bernstein and M. V. Querry in U. S. Patent 2,725,388 [*C.A.* **50**, 15605 (1956)] report the preparation of ethyl choladienate by a method similar to ours with the differences that the tosylate was not isolated and the dehydrotosylation medium was pyridine. The yield of product considered to be a mixture of the $\Delta^{2,11}$- and $\Delta^{3,11}$-isomers was about 27%.

(11) L. C. King and B. M. Regan, THIS JOURNAL, **74**, 5617 (1952).

(12) L. F. Fieser and R. Ettore, *ibid.*, **75**, 1700 (1953).

(13) K. Yamasaki, V. Rosnati, M. Fieser and L. F. Fieser, *ibid.*, **77**, 3308 (1955). We wish to thank Professor Fieser for making available to us a copy of the paper before publication and for providing comparison samples of methyl and ethyl 3-cholenate, and Dr. Wei-yuan Huang for valuable help in infrared studies.

(14) H. Wieland, K. Kraus, H. Keller and H. Ottawa, *Z. physiol. Chem.*, **241**, 47 (1936).

(15) R. T. Bridgewater and C. W. Shoppee [*J. Chem. Soc.*, 1709 (1953)] also reported an exclusive coprostene product in the pyrolysis of 3α- and 3β-coprostanyl benzoates as well as in elimination reactions on several 3β-coprostanyl halides, although their preference is for the Δ^2-structure.

(16) D. H. R. Barton and W. J. Rosenfelder, *ibid.*, 1048 (1951).

(17) J. Barnett and T. Reichstein, *Helv. Chim. Acta*, **21**, 926 (1938).

ently served only as intermediary in the synthesis of 12α-hydroxycholanic acid.

The 9(11)-cholenic acid methyl ester was conveniently prepared from the 3,9(11)-dienic compound by selective hydrogenation; the 9(11)-double bond was not hydrogenated with platinum oxide catalyst. Methyl 9(11)-cholenate had been prepared previously by Reich and Reichstein[18] and by Fieser, Heymann and Rajagopalan.[19]

Experimental[20,21]

Dehydrotosylation.—This method is used in the preparation of several compounds reported in this paper. Only the first preparation will be given in detail. In the case of the methyl esters, the procedure becomes simpler in that solid (usually crystalline) product separated from the lutidine reaction mixture on addition of ice chips. By washing the solid on a filter with ice-cold 3% hydrochloric acid, a satisfactory preliminary purification is effected.

Ethyl 3,11-Choladienate (45).—(a) Ethyl 3α-tosyloxy-11-cholenate (24),[1] 3.0 g., was refluxed for 3 hr. in 36 ml. of 2,6-lutidine (Eastman Kodak Co. practical grade). Large colorless crystals of lutidine *p*-toluenesulfonate separated on cooling the mixture to room temperature and redissolved when chips of ice were added. The mixture was transferred to a separatory funnel with ether and extracted in order with ice-cold 3% hydrochloric acid, 10% $NaHCO_3$ solution and water to neutrality. The ethereal solution was dried with Drierite and evaporated to a clear residual oil weighing 2.04 g. The oil was dissolved in purified ligroin (63–70°) and chromatographed, using alumina (Fisher) in the ratio of 30:1. ligroin (63–70°) eluted material amounting to 1.32 g. (64%) was dissolved in 95% ethanol and seeded, yielding good crystals. One crystallization gave colorless hexagonal plates with m.p. 67–68.5°, $[\alpha]_D$ + 32.4°*.

Anal. Calcd for $C_{26}H_{40}O_2$ (384.58): C, 81.20; H, 10.48. Found: C, 80.98; H, 10.72.

(b) **By Pyrolysis.**—Ethyl 3α,12α-dibenzoxycholanate (41), 5.0 g., was pyrolyzed at 200–350° for 160 min. at 28 mm. to yield 3.01 g. of oil. Chromatography of 1.78 g. of the oil gave 218 mg. of a ligroin (35–60°) eluted material of m.p. 45–54°. After eight recrystallizations, 41 mg. of colorless needles of m.p. 68–69° was obtained. A mixed m.p. with material obtained in (a) gave no depression.

Anal. Calcd. for $C_{26}H_{40}O_2$: C, 81.20; H, 10.48. Found: C, 81.44; H, 10.62.

(c) **By Esterification.**—Saponification of methyl 3,11-choladienate (46) in methanolic KOH, acidification and re-esterification of the resulting acid in absolute ethanol containing concentrated HCl gave dense, colorless prisms, m.p. 66–68°, melting point not depressed when mixed with (a).

Methyl 3,11-Choladienate (46). (a) By Dehydrotosylation.—Three grams of methyl 3α-tosyloxy-11-cholenate (23) gave 1.97 g. (95%) of colorless solid with m.p. 76–78° on addition of ice chips to the lutidine mixture. Fractional crystallization in methanol raised the melting point very gradually, although after several crystallizations the product assumed a regular flat hexagonal form. After eleven recrystallizations the top material melted 82.5–83.5°, weight 116 mg. However, by passing the crude solid dissolved in ligroin (63–70°) through an alumina column, the persistent impurity was removed and shiny flat hexagons melting at 83.5–84.5° were obtained after two crystallizations, $[\alpha]_D$ +33.4°*.

Anal. Calcd. for $C_{25}H_{38}O_2$ (370.55): C, 81.03; H, 10.34. Found: C, 80.8; H, 10.1.

(b) **By Pyrolysis.**[4]—The product of pyrolysis of 10 g. of methyl 3α,12α-dibenzoxycholanate[22] after chromatography melted 50–75° and weighed 2.54 g. After fourteen crystallizations, colorless needles melting 84–85° were obtained, mixed m.p. with product in (a), no depression.

Methyl 3,9(11)-choladienate (47) was prepared by dehydrotosylation of methyl 3α-tosyloxy-9(11)-cholenate (22)[1] in 75% yield as colorless needles when crystallized from methanol, m.p. 61–63°, $[\alpha]_D$ +21.4°*.

Anal. Calcd. for $C_{25}H_{38}O_2$ (370.55): C, 81.03; H, 10.34. Found: C, 80.9; H, 10.5.

Methyl 3-Cholenate (48).—By dehydrotosylation of methyl 3-tosyloxycholanate (methyl lithocholate tosylate) (20), a 95% yield of solid with m.p. 69–72.5° separated on addition of water to the lutidine solution. After chromatography and one crystallization in methanol, shiny, thin rectangular plates, m.p. 74.5–75°, resulted, $[\alpha]_D$ +17.0°.

Anal. Calcd. for $C_{25}H_{40}O_2$ (372.57): C, 80.59; H, 10.82. Found: C, 80.4; H, 10.8.

Admixed with authentic methyl 3-cholenate,[13] m.p. 73–74°, $[\alpha]_D$ +19°, no depression in melting point was found, and the infrared spectra of the two preparations were identical.

Ethyl 3-Cholenate (49).—(a) Dehydrotosylation of ethyl lithocholate tosylate (21) resulted in 52% yield of product melting at 76–80°, after crystallization from aqueous methanol, not chromatographed. (b) By ester interchange: Two hundred mg. of methyl 3-cholenate (48) of m.p. 74.5–75° in 16 ml. of an ethanolic HCl solution (made up by addition of 0.4 ml. of concd. HCl to 25 ml. of absolute ethanol) after standing at room temperature (25–28°) for 18 days, when diluted with water to near turbidity, afforded feathery needles melting at 80–81.5° which recrystallized from aqueous ethanol as colorless needles, m.p. 82.5–83.5°, $[\alpha]_D$ +16.7°. No depression in a mixed m.p. determination with authentic ethyl 3-cholenate[13] (m.p. 82–83°).

Anal. Calcd. for $C_{26}H_{42}O_2$ (386.60): C, 80.77; H, 10.95. Found: C, 80.5; H, 11.0.

Methyl 9(11)-cholenate (50), prepared by partial hydrogenation of methyl 3,9(11)-choladienate (47) in methanol and Adams catalyst, was crystallized from methanol, m.p. 68.5–72°. Chromatographed, it crystallized from methanol and melted at 45–49°; but from a dilute solution, separated slowly as long, colorless needles, m.p. 72–72.8°, giving a positive tetranitromethane test, $[\alpha]_D$ +34.8°*. (Reich and Reichstein's product[18] prepared by another method melted 49.5–50°, 67–67.5°; the preparation of Fieser, Heymann and Rajagopalan[19] had a m.p. of 67.4–68.2°.)

Anal.[23] Calcd. for $C_{25}H_{40}O_2$ (372.57): C, 80.59; H, 10.82. Found: C, 80.62; H, 11.00.

Methyl 11-cholenate (51) was prepared in poor yield following the pyrolysis method of Alther and Reichstein.[24] The product which recrystallized from methanol melted at 58–59°, $[\alpha]_D$ +29.0°.

Anal.[23] Calcd. for $C_{25}H_{40}O_2$ (372.57): C, 80.59; H, 10.82. Found: C, 80.44; H, 10.69. (Analysis on material dried at room temperature and 1 mm. indicates solvation with 0.5 mole of CH_3OH.)

Ethyl 11-cholenate (52) was made by hydrolysis of methyl 11-cholenate (51) to 11-cholenic acid and subsequent esterification of the acid (not crystallized) with ethanol (HCl). Crystallization from absolute ethanol first yielded needles melting at 33–34.5°. These low-melting crystals after standing in a desiccator melted 51–52° and when recrystallized retained the 52° m.p., $[\alpha]_D$ +30.8°.

Anal.[23] Calcd. for $C_{26}H_{42}O_2$: C, 80.77; H, 10.95. Found: C, 80.80; H, 10.85.

Methyl 12α-Hydroxy-3-cholenate (53).—(a) By dehydrotosylation of methyl 3α-tosyloxy-12α-hydroxycholanate (25) a product melting 102–109° was obtained in 90% yield.

(18) H. Reich and T. Reichstein, *Helv. Chim. Acta.*, **26**, 562 (1943).

(19) L. F. Fieser, H. Heymann and S. Rajagopalan, THIS JOURNAL, **72**, 2306 (1950).

(20) Microanalysis by the Microchemical Laboratory of New York University, and by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

(21) Melting points were taken on an electrical micro-hotstage and are uncorrected. Optical rotations were determined in 1–2% chloroform solutions at about 25°, except where noted, using a Schmidt and Haensch polarimeter, and values are accurate to ±1°. The values marked with an asterisk (*) were obtained with a Keston polarimeter attachment (Standard Polarimeter Co., 225 E. 54th St., N. Y. C.) to a Beckman DU spectrophotometer with accuracy estimated to be better than ±2°.

(22) B. F. McKenzie, W. F. McGuckin and E. C. Kendall, *J. Biol. Chem.*, **162**, 555 (1946).

(23) Sample melted at 80° in vacuum before analysis.

(24) H. B. Alther and T. Reichstein, *Helv. Chim. Acta*, **25**, 805 (1942).

Two crystallizations from methanol brought the m.p. to 111–112°, not raised by further recrystallization, $[\alpha]_D$ +24.0°. *Anal.* Calcd. for $C_{25}H_{40}O_3$ (388.6): C, 77.27; H, 10.38. Found: C, 77.1; H, 10.2. This was probably identical with the product of Barnett and Reichstein[17] prepared similarly but with pyridine as solvent and assigned the Δ^3-structure with no comment. Their material had m.p. 110–111° out of methanol; no rotation reported. (b) By pyrolysis of methyl desoxycholate 3-cathylate,[25] a product in 88% yield, m.p. 106.5–109° after recrystallization from methanol, was obtained.

(25) L. F. Fieser and S. Rajagopalan, THIS JOURNAL, **72**, 5530 (1950).

Ethyl 12α-Hydroxy-3-cholenate (54).—(a) By dehydrotosylation of ethyl 3α-tosyloxy-12α-hydroxycholanate (26) a 98% yield of 92–95° melting material was produced, which, when recrystallized from methanol, melted at 92–97°. When pyridine was used instead of lutidine, the yield of product dropped to 32%. (b) By ester interchange: After 13 days at room temperature in ethanolic HCl methyl 12α-hydroxy-3-cholenate (53) gave a 60% yield of product melting 96–99° out of ethanol–H_2O (2:1). After chromatography, the m.p. was 97.5–99.2°, $[\alpha]_D$ +27.0°*.

Anal. Calcd. for $C_{26}H_{42}O_3$ (402.60): C, 77.56; H, 10.52. Found: C, 77.53; H, 10.26.

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[CONTRIBUTION FROM THE DEPT. OF PATHOLOGY, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE]

Potentiometric Study of Gelatin and its Methyl Ester[1a-c]

BY SAM KATZ AND PAUL H. MAURER

RECEIVED NOVEMBER 9, 1956

The proton dissociation isotherm for gelatin was established by both titration and equilibration techniques at 37.0 ± 0.02°, 0.15 ionic strength. It was demonstrated that even though the isotherms obtained by titration were reproducible and reversible they were approximately 10% lower than those obtained by the equilibration technique. Similar curves evaluated from equilibration data were analyzed in terms of the number and dissociation constants of the constituent prototropic groups. The resulting values are in good accord with those established by amino acid analyses. A method is proposed for the quantitative determination of the degree of esterification of the methyl ester of gelatin. This is based on the selective alkaline hydrolysis of the ester linkages. The analytical values are considered accurate to 4%.

Introduction

In the course of the investigation of gelatin and some of its derivatives as possible plasma expanders, it was observed that the solubility and gelation properties of the methyl ester of gelatin were markedly different from the parent material.[2] This observation suggested that if this effect were real, *i.e.*, not due to the hydrolysis of gelatin during the esterification procedure, some information relevant to gelation might be derived.

The information derived from potentiometric titrations is important for the interpretation of the results obtained from the other physical techniques such as viscosity, osmometry, etc.[3] From these data a provisional estimate of the electrostatic charge of the gelatin as a function of *p*H may be made. Furthermore, the analysis of the dissociation isotherm will reveal not only the number and the dissociation constants of the prototropic groups but also their relative distribution as a function of *p*H. By comparison of the titration curves of the original and modified gelatins, a quantitative measure of the extent of reaction can be made. If extensive hydrolysis of the protein were to occur, this would be reflected by an increase either in the number of the carboxyl and/or amino groups.

Experimental

Materials.—The beef bone gelatin, lot #148-1B, was kindly supplied by Dr. D. Tourtellote of the Knox Gelatine Company. Analytical grade reagents were utilized without further purification. The standard solutions of NaOH (0.2 *N*) and HCl (0.2 *N*) were prepared by diluting Stansol solutions (Standard Solution Company, Menasha, Wisconsin) to the desired concentrations and restandardized before use. The NaOH was protected from the atmosphere by a tube packed with activated Alumina and Caroxite, a CO_2 absorbent. Nitrogen, water pumped, stated as being 99.6% pure was employed whenever necessary.

Prior to titration, the gelatin solutions were dialyzed at 37° in the presence of toluene against 6 changes of solvent (0.15 *M* KCl) for at least 3 days; the ratio of the gelatin (inner solution) to the solvent being 1:4. The dialysis sac was rotated during this period.

Protein concentrations were determined by dried weight at 115 ± 5°, correcting for the salt content. The validity of this correction was established by demonstrating that the dry weight of the outer solution differed from that of the 0.15 *M* KCl by less than 0.1%.

The methyl ester of gelatin (Metgel) was prepared by the procedure of Fraenkel-Conrat and Olcott.[4] The preparation was washed once with methanol and then repeatedly with acetone. The solvent was removed *in vacuo* at room temperature. The product, which was obtained in 80% yield, was identical in gross appearance to the parent substance.

Equipment.—The *p*H was determined with a Beckman Model G *p*H meter with a type E-2 all-*p*H glass electrode in conjunction with a saturated calomel electrode. The instrument was calibrated at 37° against 0.05 *M* potassium hydrogen phthalate, *p*H 4.03,[5] and at 6.98 and 9.88 using 2 standard buffers.

The iso-ionic *p*H for gelatin was established by electrodialysis employing both parchment paper membrane and Amberplex ion resin membranes (donated by Rohm and Haas).

Titration.—The standard acid or alkali was introduced with a Koch micro-buret, 5-ml. capacity, into 15.00 ml. of 2% gelatin solution contained in a 50-ml. water-jacketed beaker. The temperature was maintained at 37.0 ± 0.2°. Control experiments in which the protein was eliminated were performed in order to evaluate the activity coefficient of the reactants. Prior to the initiation of any experiment, the electrodes were standardized against two of the buffers, and upon completion of the run they were rechecked. In any case, where the drift in reading exceeded 0.03 *p*H unit the experiment was discarded. In the alkaline range of ti-

(1) (a) This investigation was supported by a grant from the Office of the Surgeon General, Department of the Army, Contract No. DA-49-007-MD-248, upon the recommendation of the Subcommittee of Shock of the National Research Council. (b) Presented in part at the 130th Meeting of the American Chemical Society, Atlantic City, New Jersey, Sept., 1956. (c) Publication No. 95 of the Department of Pathology, University of Pittsburgh School of Medicine.

(2) P. H. Maurer, *Arch. Biochem. et Biophys.*, **58**, 205 (1955).

(3) S. Katz and P. H. Maurer in preparation.

(4) H. A. Fraenkel-Conrat and H. S. Olcott, *J. Biol. Chem.*, **161**, 259 (1945).

(5) National Bureau of Standards, Letter Circular LC 933 (1948).