radical chain mechanism which starts with a single +2 choline ion and continues through about 300 ions before the chain reaction is terminated. What part, if any, excited molecules play in the decomposition mechanism, is unknown. The only facts known at present about the postulated chain reaction are: (a) the energy of activation is high enough so that the chain does not proceed at liquid nitrogen temperatures and (b) the presence of iodide (or iodine), in the case of choline iodide, also breaks the chain. One way in which the chain reaction and the chain-breaking step may be taking place is

$$[(CH_3)_3NCH_2CH_2OH]^+ \xrightarrow{-e^-} [(CH_3)_3NCH_2CH_2OH]^{++} \quad (1)$$

$$[(CH_3)_3NCH_2CH_2OH]^{++} \longrightarrow (CH_3)_3N\cdot^+ + {}^+CH_2CH_2OH$$

$${}^+CH_2CH_2OH \longrightarrow H^+ + CH_2{=}CHOH \quad (2)$$

$$CH_2{=}CHOH \longrightarrow CH_3CHO$$

$$(CH_3)_3N\cdot^+ + [(CH_3)_3NCH_2CH_2OH]^+ \longrightarrow (CH_3)_3N: + (CH_3)_3N\cdot^+ + {}^+CH_2CH_2OH \quad (3)$$

Chain-breaking step (in the case of choline iodide) (4)

$$(CH_3)_3N\cdot^+ + I^- \longrightarrow (CH_3)_3N: + I\cdot$$

Reactions (2) and (3) might then be repeated until the trimethylamine radical ion participated in some reaction other than (3), thus breaking the chain. However, since the crystal structure of choline chloride has not been determined, it is not known whether the spatial arrangement of atoms in a choline chloride crystal would be such as to favor the operation of this mechanism. It is probable that the crystal structures of the choline analogs, when they are accurately known, will do much to explain the great difference in radiation sensitivity between choline chloride and the other choline analogs.

The explanation of the great difference in the G values of the C^{14}-beta- and the 2–4 mv. electron-decomposed choline chloride may lie in the far higher flux of electrons in the case of the high energy particles; the higher energy irradiations took place during a few milliseconds (pulsed beam), the low-energy ones during weeks or months. There is, therefore, apparently an intensity effect in which the lengths of the chains are inversely related to the rate of chain starting.

By analogy with the work of Ingold's group[16] on the thermal decomposition of quaternary ammonium bases (Hofmann elimination reaction), it might be expected that trimethyl-2-chloroethylammonium chloride, with an even stronger electron-attracting group on the β-carbon atom, might be even more unstable than choline chloride. However, toward radiation decomposition the former compound is much more stable than choline chloride. Since a chain mechanism apparently is present in the case of choline chloride, but not in any of the analogs (with the possible exception of betaine hydrochloride), the radiation–decomposition reaction is quite different from the thermal decompositions and no analogies can be drawn.

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[CONTRIBUTION FROM THE PACIFIC FISHERIES EXPERIMENTAL STATION]

Marine Sterols. I. Isolation of 24-Methylenecholesterol from Molluscs

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A new sterol, 24-methylenecholesterol, has been isolated by a chromatographic separation of the azoyl esters prepared from the sterols of the oyster (*Ostrea gigas*) and the clam (*Saxidomus giganteus*). Oyster and clam sterols contain 36 and 53% respectively, of this sterol.

It has long been recognized that oysters contain sterols other than cholesterol.[1,2] Ostreasterol has been isolated from several molluscs including the oyster, *Ostrea virginica*.[2] There have been several structures proposed for this sterol but the most recent evidence indicates that it is identical with chalinasterol($\Delta^{5,22(23)}$-campestadiene-3β-ol).[2–4] However, the evidence appears to be based solely on a resemblance of physical properties, a frequently unreliable criterion when applied to sterols. Ostreasterol was first isolated as the most insoluble steryl acetate from *Ostrea virginica* and later it was purified further *via* the tetrabromide.[2,3]

In the present investigation 24-methylenecholesterol was isolated by chromatography of the steryl azoates prepared from the sterols of the oyster (*Ostrea gigas*) and the butter-clam (*Saxidomus giganteus*), on silicic acid. This sterol does not have the same properties as ostreasterol, for when the mixed acetates from *Ostrea gigas* were recrystallized repeatedly, the most insoluble acetate was still a mixture composed of 29% 24-methylenecholesterol and 71% of chromatographic zone 3. Thus judging from the original composition, zone 3 and not zone 2 (24-methylenecholesterol) forms the more insoluble acetate. Zone 3 has been shown to

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(2) W. Bergmann, *J. Biol. Chem.*, **104**, 317 (1934).

(3) W. Bergmann, *ibid.*, **104**, 553 (1934).

(4) W. Bergmann and E. M. Low, *J. Org. Chem.*, **12**, 67 (1947).

contain two double bonds by hydrogenation and perbenzoic acid titration. Thus, since there are at least two disunsaturated oyster sterols, purification *via* the tetrabromides does not appear to be nearly so desirable as the present chromatographic separation. The infrared spectra clearly show that neither zone 2 nor zone 3 sterol contains a *trans*-22,-23-bond (as does chalinasterol = ostreasterol) because of the absence of a strong band at 10.3 μ (970 cm.$^{-1}$).[5] The absence of either a *cis*- or *trans*-22,-23-bond is demonstrated further by the failure of either zone to yield methylisopropylacetaldehyde upon ozonolysis under conditions where good yields were obtained from ergosterol.

The sterol from chromatographic zone 2 has the same parent nucleus as campesterol. Zone 2 was reduced to the stanol with Adams catalyst in glacial acetic acid and the optical rotations of the stanol and derivatives were compared to those of campestanol (Table I). The saponification equivalent of zone 2 acetate furnishes additional evidence for the C_{28} skeleton.

TABLE I

PROPERTIES OF ZONE 2 STANOL

	Zone 2		Campestanol	
	M.p., °C.	$[\alpha]^{22}D$	M.p., °C.	$[\alpha]^{22}D$
Acetate	144	+18.2°	144	+18.3°
m-Dinitro-bz.	199	+22.4	199	+22
Stanol	145	+32.6	146	+32
Sapn. equiv.	Calcd. 441		Found 445	

The infrared spectra of zone 2 and the optical rotational differences of its derivatives definitely establish the presence of a double bond at the 5-position. This is confirmed by the reaction rate of the sterol when treated with the modified Liebermann–Burchard reagent.[6,7]

The isolation of formaldehyde in good yield (50%) by the ozonolysis of zone 2 establishes that the additional double bond must be at the 20,21-, 24,28- or 25,26-position. The solid film infrared spectra of zone 2 sterol differs from that of campesterol and other Δ^5-sterols by the presence of a strong band at 890 cm.$^{-1}$ and a band of medium strength at 1640 cm.$^{-1}$. This evidence confirms the structure CH_2=CRR.[1] A shoulder at 1362 cm.$^{-1}$ indicates the presence of the *gem*-dimethyl group at C_{25} and the band at 1330 cm.$^{-1}$ is quite possibly due to the CH group at C_{25}.[8] Further, the decomposed ozonide from a 25(26)-bond would yield a methyl ketone and the product of such treatment gave no iodoform reaction. Thus the absence of a 25,26-bond is indicated.

Conclusive proof that the new sterol is 24-methylenecholesterol was obtained by the isolation of 24-ketocholesterol from the ozonolysis products. This sterol has been synthesized[9] previously and also isolated from the products of the ozonolysis of fucosterol ($\Delta^{5,24(28)}$-stigmastadiene-3β-ol).[10]

The abundance of C_{24}-epimeric sterols in nature is well recognized. The origin of these epimers can be explained by considering them as biological reduction products of a C_{24}–C_{28} unsaturated precursor. Indeed, fucosterol, the corresponding sterol in the C_{29}-series, has been isolated from algae.[11,12] The isolation of 24-methylenecholesterol explains the origin of C_{24}-epimers in the C_{28}-series and strengthens the biological reduction hypothesis.

Bergmann[13] has suggested that haliclonasterol and palysterol may be C_{20}-epimers on the basis of molecular rotational data and the present status of C_{24}–C_{28} unsaturated compounds suggests that a 20-methylenecholesterol also may occur in nature.

Experimental[14]

Preparation of Crude Sterols.—In a typical preparation of oyster sterols 8.75 lb. of fresh oysters were extracted with acetone in a Soxhlet for 48 hours. The acetone was removed and the fat (88 g., 2.22%) extracted into ether. The fat then was taken up in acetone and the phosphatides (6.7 g.) filtered off. The fat was saponified in the usual manner with alcoholic KOH and the non-saponifiable portion (10.9%) was extracted into ether. The sterols were precipitated with digitonin from an alcoholic solution of the non-saponifiables in the usual manner (18.9 g. digitonides). The digitonides were decomposed with pyridine[15] and the sterols crystallized from methanol (theory 4.72 g., 52% of non. sap.), m.p. 135–138°, $[\alpha]^{22}D$ −41.50°. The mixed sterols in alcohol showed maxima in the ultraviolet at 265, 271.5, 281 and 293 mμ. The intensities of these bands indicated the presence of 9.05% of $\Delta^{5,7}$-sterol (calcd. as ergosterol). The modified Liebermann–Burchard reaction indicated approximately the same amount of "fast-acting" sterol, thus excluding the possibility of any significant amounts of Δ^7, $\Delta^{8(9)}$-, $\Delta^{8(14)}$- or $\Delta^{14(15)}$-sterols in the mixture.[7,16]

Clam sterols were obtained by an identical procedure and the yields were: fat, 2.01%; acetone insoluble, 37.6% (filtered off) and non-saponifiable, 17.3% of the fat; sterol, 65.6% of the non-saponifiables calculated on the weight of digitonides.

Chromatography.—Azoyl esters were chromatographed on silicic acid:Celite, using 5.5:1 Skelly C–benzene developer exactly as previously described for the separation of plant and animal sterols.[7,17,18] In the case of oyster sterols the upper zone azoyl ester (zone 1) represents 5.02% of the total and is completely separated from zone 2 (36.13%) which in turn completely separated from zone 3 (28.55%) and zone 4 (30.30%). There is no sharp separation between zone 3 and 4 but the difference in color intensities permits an arbitrary cut of the extruded column.

The appearance of the chromatogram of clam steryl azoates was qualitatively identical to that of the oyster esters. Quantitatively there is considerably more zone 2 (53.0%) in clam than in oyster. The other zones were: (1) 3.0%; (3) 21.3% and (4) 22.7%.

Purification of Zone 2 Oyster Sterol.—The azoyl ester crystallized from benzene–ethanol in fluffy needles, m.p. 193°. The ester was hydrolyzed as previously described and the acetate prepared.[17] The acetate was treated with a tenfold excess of maleic anhydride in boiling xylene for 18 hours and the provitamin D removed as previously described.[18]

The acetate of the "provitamin D-free" sterol was prepared and this reached a constant m.p. 136° after two

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crystallizations from ethanol. Pure zone 2 sterol, prepared by hydrolysis of the acetate, melted at 142°, $[\alpha]^{22}D$ −35.0° (26 mg. in 2 ml. of $CHCl_3$).

Anal. Calcd. for $C_{28}H_{46}O$: C, 84.35; H, 11.63. Found C, 84.40; H, 11.62.

Derivatives.—The acetate of zone 2 sterol crystallized from ethanol in plates, m.p. 136°, $[\alpha]^{22}D$ −42.4° (24 mg. in 2 ml. of $CHCl_3$).

Anal. Calcd. for $C_{30}H_{48}O_2$: C, 81.76; H, 10.98. Found: C, 81.60; H, 10.84.

The benzoate crystallized from acetone in plates, m.p. 148° (clear 153°), $[\alpha]^{29}D$ −14.1° (25 mg. in 2 ml. of $CHCl_3$).

Anal. Calcd. for $C_{35}H_{50}O_2$: C, 83.61; H, 10.02. Found: C, 83.41; H, 10.10.

Molecular rotational differences: Δ^5-sterols: Δ^1 −35 ± 16, Δ^2 +81 ± 16°; zone 2 sterol: Δ^1 −46; Δ^2 +70.

Reduction of Zone 2 Acetate.—In glacial acetic acid the reduction of 30.0 mg. of the acetate was 95% complete for two double bonds on shaking for 7 minutes with 20 mg. of pre-reduced Adams catalyst and the theoretical amount of 3.05 ml. was taken up within 20 minutes. The hydrolyzed reduction product crystallized from methanol in plates, m.p. 145°, $[\alpha]^{22}D$ +32.6° (12 mg. in 1 ml. of $CHCl_3$). The melting point was not depressed by authentic campestanol, m.p. 145°. The product gave no color with the Liebermann–Burchard reagent and showed no absorption at 6 μ in the infrared due to C=C stretching.

Anal. Calcd. for $C_{28}H_{50}O$: C, 83.51; H, 12.51. Found: C, 83.62; H, 12.63.

Derivatives.—The acetate was crystallized from ethanol, m.p. 144°, $[\alpha]^{22}D$ +18.2° (28 mg. in 2 ml. of $CHCl_3$).

Anal. Calcd. for $C_{30}H_{52}O_2$: C, 81.02; H, 11.79. Found: C, 80.93; H, 11.75.

m-Dinitrobenzoyl chloride (120 mg.), 30 mg. of zone 2 stanol and 1 ml. of pyridine were warmed on a steam-bath for 2 hours. Ten volumes of ice-cold methanol were added to the chilled mixture and the product washed with cold methanol–water. The *m*-dinitrobenzoate crystallized from benzene–ethanol in fluffy needles, m.p. 199°, mixed m.p. with authentic campestanyl *m*-dinitrobenzoate 199°, $[\alpha]^{22}D$ +22.4° (18 mg. in 2 ml. of $CHCl_3$).

Anal. Calcd. for $C_{35}H_{52}O_6N_2$: C, 70.41; H, 8.78. Found: C, 70.36; H, 8.82.

Saponification Equivalent.—Zone 2 acetate (0.800 g.) was saponified in 10.00 ml. of 0.4846 *N* alcoholic NaOH for 1 hour and the residual alkali back-titrated with 5.554 ml. of 0.5488 *N* HCl.

Anal. Calcd. for $C_{30}H_{48}O_2$: mol. wt., 440.7. Found: mol. wt., 445.

Zone 2 Clam Sterol.—This sterol was isolated and purified in an identical manner to zone 2 oyster sterol. The melting point, optical rotation, modified Liebermann–Burchard reaction and infrared spectra were identical to those of the oyster sterol. The properties of all derivatives were likewise identical.

Ozonolysis.—Seven hundred mg. of zone 2 acetate from clam was taken up as a suspension in 15 ml. of glacial acetic acid and ozonized. The exit gases were scrubbed in a trap containing 40 ml. of ice-cold water. This trap, in turn, was connected to an indicator bottle containing acidified KI. The ozonolysis was stopped after 20 minutes, by which time the KI solution had taken on a light brown color. The acetic acid and wash water were then combined and steam distillation was carried out into a solution of dimedone. The *p*H of the solution was maintained at 5.8 by the addition of alkali and distillation was continued until the formation of dimedone derivative was negligible.

The yield of dimedone derivative, m.p. 187°, was 234 mg. (50.4% of theory for one methylene group). It crystallized from methanol–water in fluffy needles, m.p. 190°, undepressed on admixture with authentic formaldehyde dimedone.

Anal. Calcd. for $C_{17}H_{24}O_4$: C, 69.82; H, 8.27. Found: C, 69.87, 69.94; H, 8.24, 8.30.

24-Ketocholesteryl Acetate by Ozonolysis.—Two hundred and forty mg. of zone 2 acetate from oyster was dissolved in 50 ml. of acetic acid (3 times distilled over chromic oxide) held at 17°, and ozone was admitted at a rate of 1.68 mg. per minute. Ozonolysis was continued for 13.8 minutes (theory for 90% saturation for 1 double bond). The acetic acid solution was diluted with 150 ml. of water and the formaldehyde distilled off and trapped in dimedone (87 mg. identified as previously described). The aqueous acid solution was extracted with chloroform and the chloroform was washed with sodium carbonate followed by water and the sterol residue obtained by distillation *in vacuo*. The residue was taken up in benzene and chromatographed on silicic acid–Celite.[7] The first material off the column (25 mg.) was unchanged zone 2 steryl acetate, m.p. 136°. The column was then washed with benzene containing 2% chloroform and on evaporation of the solvent a solid residue, 130 mg., m.p. 126–127°, was obtained. Recrystallization from ethanol gave 24-ketocholesteryl acetate, m.p. 127°, $[\alpha]^{22}D$ −43° (Hey, *et al.*, report 127–128°, $[\alpha]^{15}D$ −43°[10]). Synthetic 24-ketocholesterol acetate gave mixed m.p. 127°.

Anal. Calcd. for $C_{29}H_{46}O_3$: C, 76.68; H, 10.48. Found: C, 76.57; H, 10.41.

The polyoxygenated sterol remaining on the column required ether for elution.

24-Ketocholesteryl Acetate 2,4-Dinitrophenylhydrazone.—The 2,4-dinitrophenylhydrazone crystallized from ethanol had m.p. 170°. The derivative prepared from synthetic 24-ketocholesteryl acetate, m.p. 170°, gave mixed m.p. 170°.

Anal. Calcd. for $C_{35}H_{50}O_6N_4$: C, 67.50; H, 8.09. Found: C, 67.49; H, 7.98.

24-Ketocholesteryl Acetate Oxime.—A solution of 15 mg. of 24-ketocholesteryl acetate and 15 mg. of hydroxylamine hydrochloride in 4 drops of anhydrous pyridine and 4 drops of absolute ethanol were warmed on the steam-bath for 1.7 hours. The addition of water precipitated the oxime, m.p. 168°. Repeated recrystallization from aqueous ethanol did not raise the melting point above 169° (Riegel and Kaye report 155–158.5°[9]). Melting point of the oxime of synthetic 24-ketocholesteryl acetate 169°, mixed m.p. 169°.

Anal. Calcd. for $C_{29}H_{47}O_3N$: C, 76.10; H, 10.35; N, 3.06. Found: C, 76.22; H, 10.46; N, 2.95.

24-Ketocholesterol.—The acetate was hydrolyzed and 24-ketocholesterol crystallized from aqueous ethanol had m.p. 137°, $[\alpha]^{20}D$ −37° (137–138.5°, $[\alpha]^{25}D$ −37°[9]); mixed m.p. with synthetic 24-ketocholesterol 137°.

Anal. Calcd. for $C_{27}H_{44}O_2$: C, 80.94; H, 11.07. Found: C, 80.86; H, 11.03.

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