Nuclear magnetic resonance and infrared spectra of Δ^{24}- and C-24 saturated steroids

TERENCE J. SCALLEN* and WILLIAM KRUEGER‡

Departments of Biochemistry and Medicine, University of Minnesota Medical School, Minneapolis, Minnesota 55455, and Department of Biochemistry, University of New Mexico, School of Medicine, Albuquerque, New Mexico 87106

ABSTRACT The infrared (IR) and nuclear magnetic resonance (NMR) spectra of eight Δ^{24}-steroids and nine C-24 saturated steroids were examined. NMR spectra allow unambiguous assignment of the biologically important Δ^{24}-bond; introduction of a Δ^{24}-bond causes the appearance of peaks at δ 1.60 and 1.68 associated with the C-26, C-27 isopropylidene methyls, while C-24 saturated steroids of the cholestane series possess peaks at δ 0.82 and 0.91 associated with the C-26, C-27 *gem*-dimethyls. IR spectra show a good correlation between the introduction of a Δ^{24}-bond and a marked decrease in intensity of a band at 1365 cm^{-1}. NMR and IR spectra also allow an inference about the presence and location of nuclear double bonds in Ring B of cholesterol precursors.

KEY WORDS sterols · steroids · cholesterol · biosynthesis · infrared · nuclear magnetic resonance · spectra

In studies of sterol[1] intermediates in cholesterol biosynthesis a vast array of structural possibilities exist (1). Specifically, carbon–carbon double bonds have been encountered in cholesterol precursors at the following locations of the steroid molecule: Δ^5, Δ^7, Δ^8, $\Delta^{5,7}$ and Δ^{24} (2–6). Furthermore, when it is possible to isolate these sterols, often only a few milligrams are available for structural analysis. Although chemical oxidative methods can be valuable for the determination of double bond location in steroids (7,8) these methods have certain disadvantages: (*a*) relatively large amounts of material are needed, (*b*) the material used in the oxidation is consumed in the process of analysis, and (*c*) unless yields are quantitative or internal standards of known purity are employed, it is not possible to detect impurities which lack unsaturation.

All known pathways for cholesterol formation must at some stage include Δ^{24}-sterols as intermediates. Unfortunately, methods for the demonstration of this biologically important Δ^{24}-bond in small amounts of material have been particularly unsatisfactory. In fact, assignment of the Δ^{24}-bond has been made only on the basis of biogenetic considerations and chromatographic mobility (9).

For these reasons it seemed desirable to systematically examine the nuclear magnetic resonance (NMR) and infrared (IR) spectra of several Δ^{24}- and C-24 saturated sterols and to attempt structural correlations where possible. Our findings show that NMR spectra can unambiguously assign the Δ^{24}-bond in sterols of the cholestane series. Also, the presence and location of the nuclear double bonds may be inferred from NMR and IR spectra.

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Abbreviation: GLC, gas–liquid chromatography.

* Please address all requests for reprints to: Department of Biochemistry, University of New Mexico, School of Medicine, Albuquerque, N. Mex. 87106.

‡ Present address: The Upjohn Co., Kalamazoo, Mich.

[1] Names of steroids used in this paper are: Δ^5-cholestenol, cholest-5-en-3β-ol (cholesterol); $\Delta^{5,24}$-cholestadienol, cholesta-5,24-dien-3β-ol (desmosterol); Δ^7-cholestenol, 5α-cholest-7-en-3β-ol (lathosterol); $\Delta^{7,24}$-cholestadienol, 5α-cholesta-7,24-dien-3β-ol; $\Delta^{5,7}$-cholestadienol, cholesta-5,7-dien-3β-ol (7-dehydrocholesterol); $\Delta^{5,7,24}$-cholestatrienol, cholesta-5,7,24-trien-3β-ol; Δ^8-cholestenol, 5α-cholest-8-en-3β-ol (zymostenol); $\Delta^{8,24}$-cholestadienol, 5α-cholesta-8,24-dien-3β-ol (zymosterol); 4α-methyl-Δ^7-cholestenol, 4α-methyl-5α-cholest-7-en-3β-ol (methostenol); cholestanol, 5α-cholestan-3β-ol; Δ^{24}-cholestenol, 5α-cholest-24-en-3β-ol; cholestanone, 5α-cholestan-3-one; Δ^{24}-cholestenone, 5α-cholest-24-en-3-one; Δ^4-cholestenone, cholest-4-en-3-one; $\Delta^{4,24}$-cholestadienone, cholesta-4,24-dien-3-one; $\Delta^{4,6}$-cholestadienone, cholesta-4,6-dien-3-one; $\Delta^{4,6,24}$-cholestatrienone, cholesta-4,6,24-trien-3-one.

MATERIALS AND METHODS

IR Spectra

Spectra were recorded by means of a Perkin-Elmer 521 double-focusing grating instrument except for those of two compounds, Δ^{8}-cholestenol and $\Delta^{8,24}$-cholestadienol, which were recorded on either a Perkin-Elmer 12-C with microscope attachment (10) or a Perkin-Elmer 237 spectrometer. KBr micropellets were employed (11). Sample sizes ranged from 50 μg to 1 mg. No evidence of polymorphism was observed when different preparations of the same compound were examined. Solution spectra (CCl_4 and CS_2) of Δ^{5}-cholestenol and $\Delta^{5,24}$-cholestadienol were essentially identical with spectra obtained using KBr pellets.

The following compounds were prepared by chemical synthesis as previously described (12): $\Delta^{4,24}$-cholestadienone, $\Delta^{4,6,24}$-cholestatrienone, and $\Delta^{5,7,24}$-cholestatrienol. Δ^{5}-Cholestenol was purified via the dibromide (13). Cholestanone, Δ^{24}-cholestenone, and Δ^{24}-cholestenol were prepared by chemical synthesis (14).

Cholestanol was purchased from Chemed, Inc., Odenton, Md. and recrystallized from anhydrous methanol, mp 141°C. Assay by GLC and solubility studies showed no contaminating cholesterol (14). Δ^{4}-Cholestenone and $\Delta^{4,6}$-cholestadienone were also purchased from Chemed, Inc. Δ^{7}-Cholestenol was purchased from Steraloids, Inc., Flushing, N.Y., and recrystallized from methanol, mp 120–121°C. $\Delta^{5,7}$-Cholestadienol was purchased from Mann Research Labs. Inc., New York, and recrystallized from acetone–methanol, mp 150–151°C. $\Delta^{5,24}$-cholestadienol was purchased from Organon Inc., West Orange, N.J., mp 120–121°C; assay by GLC (15), 95% pure.

Δ^{8}-Cholestenol was synthesized by Dr. G. J. Schroepfer, Jr. (16). $\Delta^{8,24}$-Cholestadienol, mp 109–110°C, was isolated (I. D. Frantz, Jr., T. J. Scallen, and A. N. Nelson, unpublished work) from yeast and from the skins of rats treated with triparanol.[2] $\Delta^{7,24}$-Cholestadienol was isolated from the skins of triparanol-treated rats (17), mp 102–103°; assay by silicic acid (18) and GLC (15), 98% pure. 4α-Methyl-Δ^{7}-cholestenol was prepared by chemical synthesis (19).

NMR Spectra

For cholesterol and desmosterol the spectra were obtained by using a Varian HR-60 spectrometer operating at 56.4 mc. The remaining spectra were obtained either with a Varian A-60 (University of Minnesota) or with a Varian A-60A spectrometer (University of New Mexico) operating at 60 mc. Samples were routinely run with $CDCl_3$ as solvent and 0.3% tetramethylsilane as internal reference. Sample sizes ranged from 5 to 50 mg.

[2] Triparanol (MER-29) is 1-[*p*-(β-diethyl aminoethoxy) phenyl]-1-(*p*-tolyl)-2-(*p*-chlorophenyl) ethanol.

RESULTS

Nuclear Magnetic Resonance

Detection of Δ^{24}-bond. Fig. 1 shows the NMR spectrum of $\Delta^{7,24}$-cholestadienol that was isolated from the skins of triparanol-treated rats (17). Assignments were made by employing authentic comparison sterols (e.g., Δ^{7}-cholestenol and several Δ^{24}-sterols prepared by chemical synthesis) and by the use of data presented by Bhacca and Williams (20). The following proton assignments can be made: δ 0.54 (C-18 methyl); 0.813 (C-19 methyl); 0.916, 1.00 (C-21 methyl); 1.60, 1.67 (C-26, C-27 isopropylidene methyls); 4.98 (C-24 proton); 5.15 (C-7 proton). Integration of the olefinic proton region (δ 4.7–5.3 in this case) reveals two olefinic protons.

The spectrum of Δ^{7}-cholestenol is similar to that of $\Delta^{7,24}$-cholestadienol but with three important differences: (*a*) a doublet occurs at δ 0.81, 0.92 associated with the C-26, C-27 *gem*-dimethyls; (*b*) integration of the olefinic proton region reveals one olefinic proton; and (*c*) no doublet is present at δ 1.60 and 1.67.

Table 1 shows the chemical shifts (δ) for the C-26, C-27 methyl protons for seven Δ^{24}-steroids and three C-24 saturated sterols. It is clear that a pronounced and predictable downfield shift occurs in the resonant frequencies of the C-26, C-27 methyl protons with the introduction of a Δ^{24}-bond.

Detection and Location of Nuclear Double Bonds. The presence and location of nuclear unsaturation in Ring B can be inferred from the NMR spectra by calculations similar to those described by Zürcher (21) for the C-18 and C-19 methyl proton frequencies. These calculations are based upon the assumption that the frequency shifts induced by several different functional groups are approximately additive. The calculated and observed

TABLE 1 CHEMICAL SHIFTS (δ) FOR THE C-26, C-27 METHYL PROTONS OF Δ^{24}-STEROIDS AND C-24 SATURATED STEROIDS

Steroids	δ
Δ^{24}-Steroids	
$\Delta^{5,24}$-Cholestadienol	1.57, 1.65
$\Delta^{7,24}$-Cholestadienol	1.60, 1.67
$\Delta^{8,24}$-Cholestadienol	1.62, 1.69
$\Delta^{5,7,24}$-Cholestatrienol	1.62, 1.70
Δ^{24}-Cholestenol	1.58, 1.67
Δ^{24}-Cholestenone	1.61, 1.65
$\Delta^{4,24}$-Cholestadienone	1.60, 1.68
C-24 Saturated steroids	
Δ^{5}-Cholestenol	0.81, 0.92
Δ^{7}-Cholestenol	0.81, 0.92
$\Delta^{5,7}$-Cholestadienol	0.82, 0.93

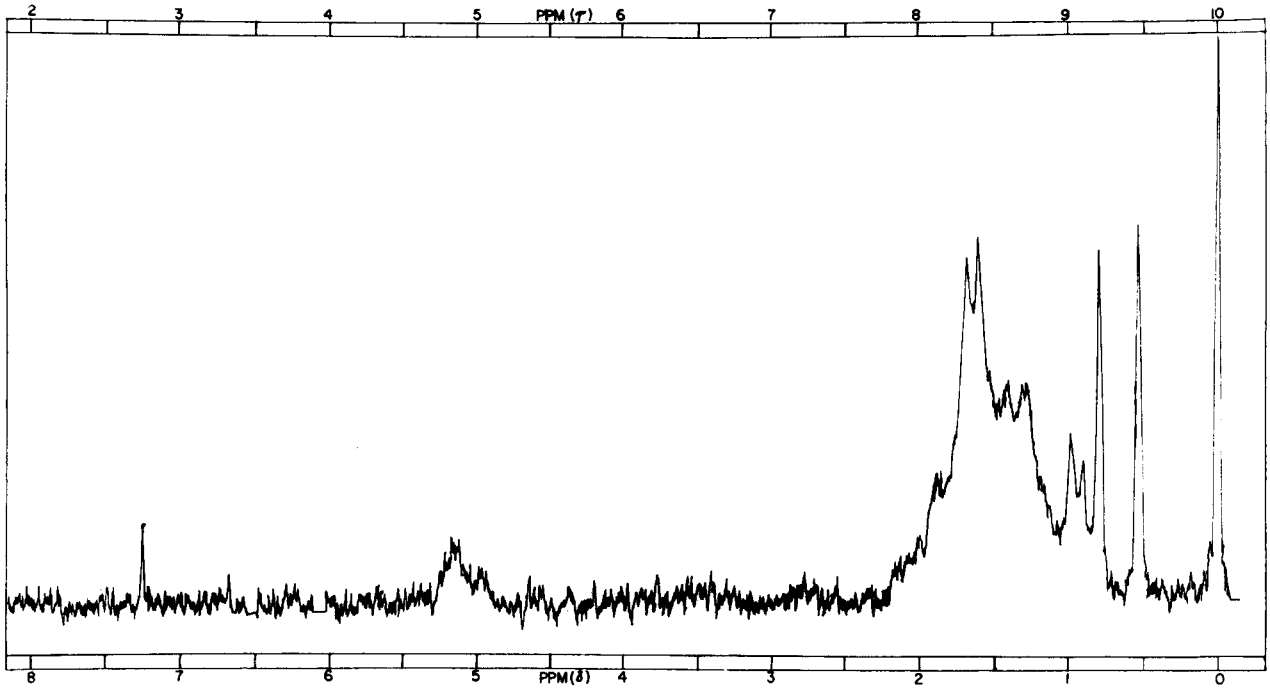

FIG. 1. NMR spectrum of $\Delta^{7,24}$-cholestadienol.

C-19 and C-18 methyl proton frequencies for several steroids are presented in Table 2. The table shows that the presence or absence of a Δ^{24}-bond has little or no effect upon the resonant frequencies of the C-18 or C-19 methyls, and that an excellent correlation exists between calculated and observed chemical shifts for different nuclear double bond locations, except possibly for the C-18 methyl protons for zymosterol ($\Delta^{8,24}$-cholestadienol).

Infrared

1300–1400 cm^{-1}. This region has been assigned to methyl and methylene bending vibrations (22, 23). Fig. 2 compares five pairs of compounds which differ only in the presence or absence of a Δ^{24}-bond. The band seen at 1365 cm.$^{-1}$ (1361–1368 cm.$^{-1}$) in the C-24 saturated compounds is clearly decreased in relative intensity [when compared with its higher frequency companion (1373–1378 cm^{-1})] with the introduction of a Δ^{24}-bond. For Δ^{8}-cholestenol (3a) and $\Delta^{8,24}$-cholestadienol (3b) the band present at 1368 cm^{-1} is seen only as a poorly defined shoulder; thus, in this case, the difference between these two sterols is not as clear-cut. This is possibly related to the slightly higher frequency of the band (as compared with the other examples cited) and also to lower resolution.[3] It is interesting to note that the band present at 1365 cm^{-1} in the spectrum of cholestanol (5a) was absent from that of Δ^{24}-cholestenol (5b).

TABLE 2 CALCULATED AND OBSERVED CHEMICAL SHIFTS (δ) FOR THE C-18 (18-H) AND C-19 (19-H) METHYL PROTONS OF Δ^{24}-STEROIDS AND C-24 SATURATED STEROIDS

	19-H		18-H	
	Calc.*	Obs.	Calc..	Obs.
Δ^{5}-Cholestenol	1.016	0.99	0.684	0.680
$\Delta^{5,24}$-Cholestadienol	1.016	1.00	0.684	0.68
Δ^{7}-Cholestenol	0.800	0.804	0.533	0.54
$\Delta^{7,24}$-Cholestadienol	0.800	0.813	0.533	0.554
$\Delta^{5,7}$-Cholestadienol	0.950	0.95	0.625	0.63
$\Delta^{5,7,24}$-Cholestatrienol	0.950	0.955	0.625	0.637
$\Delta^{8,24}$-Cholestadienol	0.933	0.957	0.567†	0.624
Δ^{24}-Cholestenol	0.808	0.79	0.65	0.64
Δ^{24}-Cholestenone	1.017	0.99	0.684	0.665
$\Delta^{4,24}$-Cholestadienone	1.192	1.180	0.717	0.720

* Calculated according to the method of Zürcher (21). An example of this calculation for $\Delta^{5,7,24}$-cholestatrienol follows:

	19-H	18-H
5α, 14α-Androstane	0.792	0.692
3β-OH	+0.033	+0.008
$\Delta^{5,7}$	+0.142	−0.025
17β-C_8H_{15}	−0.017	−0.050
Calc.	0.950	0.625
Obs.	0.955	0.637

† Value based on one compound. Experimental values for two additional Δ^{8}-compounds show 18-H at δ 0.595–0.608 (30).

[3] These two spectra were recorded using either a Perkin-Elmer 12-C or 237 spectrometer which are lower resolution instruments than the Perkin Elmer 521 used to record the remaining spectra.

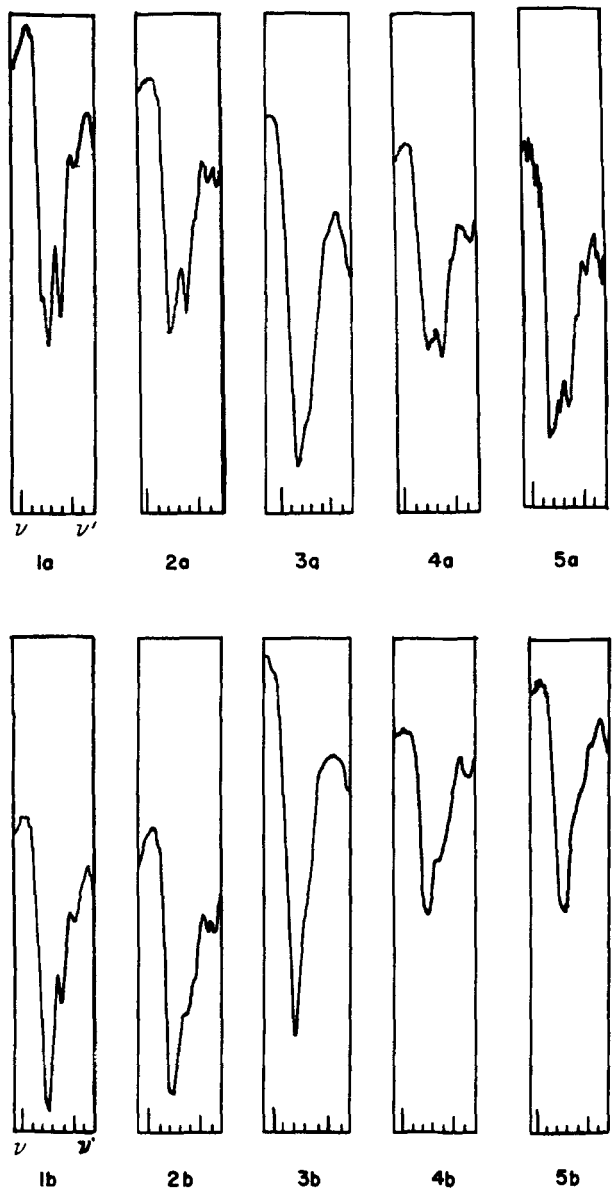

FIG. 2. IR spectra of sterols from $\nu = 1400$ cm^{-1} to $\nu' = 1340$ cm^{-1}. Those compounds shown in the top row (*a* series) are saturated at C-24; those in the bottom row (*b* series) are Δ^{24}-sterols; 1a, Δ^{5}-cholestenol; 1b, $\Delta^{5,24}$-cholestadienol; 2a, Δ^{7}-cholestenol; 2b, $\Delta^{7,24}$-cholestadienol; 3a, Δ^{8}-cholestenol; 3b, $\Delta^{8,24}$-cholestadienol; 4a, $\Delta^{5,7}$-cholestadienol; 4b, $\Delta^{5,7,24}$-cholestatrienol; 5a, cholestanol; 5b, Δ^{24}-cholestenol.

Three pairs of ketones were studied: cholestanone and Δ^{24}-cholestenone, Δ^{4}-cholestenone and $\Delta^{4,24}$-cholestadienone, and $\Delta^{4,6}$-cholestadienone and $\Delta^{4,6,24}$-cholestatrienone. In these ketones two bands, one at 1370 cm^{-1} (1367–71) and the other at 1362 cm^{-1} (1359–64) were reduced in intensity with the introduction of a Δ^{24}-bond.

800–900 cm^{-1}. The region from 800 to 900 cm^{-1} has been assigned to the out-of-plane bending vibrations of hydrogen atoms attached to doubly-bonded carbon (22, 23). Fig. 3 and Table 3 compare several Δ^{24}-sterols and the C-24 saturated counterparts. Cholestanol (1c), which has no double bonds, shows only weak absorption in this region. Δ^{24}-Cholestenol (1d) shows a small band at 824 cm^{-1}, associated with the C-24 hydrogen, not previously present in cholestanol. Fig. 3 shows that for the Δ^{5}-, Δ^{7}-, and $\Delta^{5,7}$-sterols studied, a good correlation exists between the observed spectrum and the location of the nuclear double bond. Introduction of a 4α-methyl group does have some influence upon the spectrum [compare Δ^{7}-cholestenol (3c) and 4α-methyl-Δ^{7}-cholestenol (5c)]; however, the two major bands seen in 3c at 826 and 842 cm^{-1} are also seen in 5c at 827 and 845 cm^{-1}. Interestingly, the spectra of Δ^{24}-sterols and the C-24 saturated analogues are only slightly different in this region.

1000–1100 cm^{-1}. Fig. 4 and Table 4 compare the 1000–1100 cm^{-1} region for several Δ^{24}-sterols and the C-24 saturated counterparts. Major bands in this region have been assigned to the C–O stretching vibration of the C-3 hydroxyl group (24–27). Introduction of a Δ^{24}-bond [e.g., compare cholestanol (1e) and Δ^{24}-

TABLE 3 IR SPECTRA OF Δ^{24}-STEROLS AND OF C-24 SATURATED STEROLS IN THE 800–900 CM^{-1} REGION

Compound	Fig. 3	Frequency
		cm^{-1}
Cholestanol	1c	842, 856, 886, 901 (all w)*
Δ^{24}-Cholestenol	1d	*824*,† 840, 854, 882, 900
Δ^{5}-Cholestenol	2c	*797*, *826* (sh),‡ *839*, 880
$\Delta^{5,24}$-Cholestadienol	2d	*797*, *824*, *838*, 882
Δ^{7}-Cholestenol	3c	792, *826*, *842*, 868
$\Delta^{7,24}$-Cholestadienol	3d	794, *827*, *845*, 869
$\Delta^{5,7}$-Cholestadienol	4c	799, *832*, 870 (w)
$\Delta^{5,7,24}$-Cholestatrienol	4d	799, *831*, 884 (w)
4α-Methyl-Δ^{7}-cholestenol	5c	800 (w), 817, *827*, *845*, 873

* w, weak.
† Italics denote the most intense bands in this region.
‡ sh, shoulder.

TABLE 4 IR SPECTRA OF Δ^{24}-STEROLS AND OF C-24 SATURATED STEROLS IN THE 1000–1100 CM^{-1}

Compound	Fig. 4	Frequency
		cm^{-1}
Cholestanol	1e	*1042*, *1077**
Δ^{24}-Cholestenol	1f	*1041*, *1076*
Δ^{5}-Cholestenol	2e	*1020*, *1053*
$\Delta^{5,24}$-Cholestadienol	2f	*1020*, *1055*
Δ^{7}-Cholestenol	3e	1016, *1039*, *1049*, 1094
$\Delta^{7,24}$-Cholestadienol	3f	1017, *1040*, *1050*, 1096
Δ^{8}-Cholestenol	4e	1011, 1023, *1047*, *1057*, 1105
$\Delta^{8,24}$-Cholestadienol	4f	1010, 1023, *1046*, *1056*, 1102
$\Delta^{5,7}$-Cholestadienol	5e	*1038*, *1062*
$\Delta^{5,7,24}$-Cholestatrienol	5f	*1040*, *1064*
4α-Methyl-Δ^{7}-cholestenol	6e	1004 (sh),† *1017*, *1046*, *1054*

* Italics denote the most intense bands in this region.
† sh, shoulder.

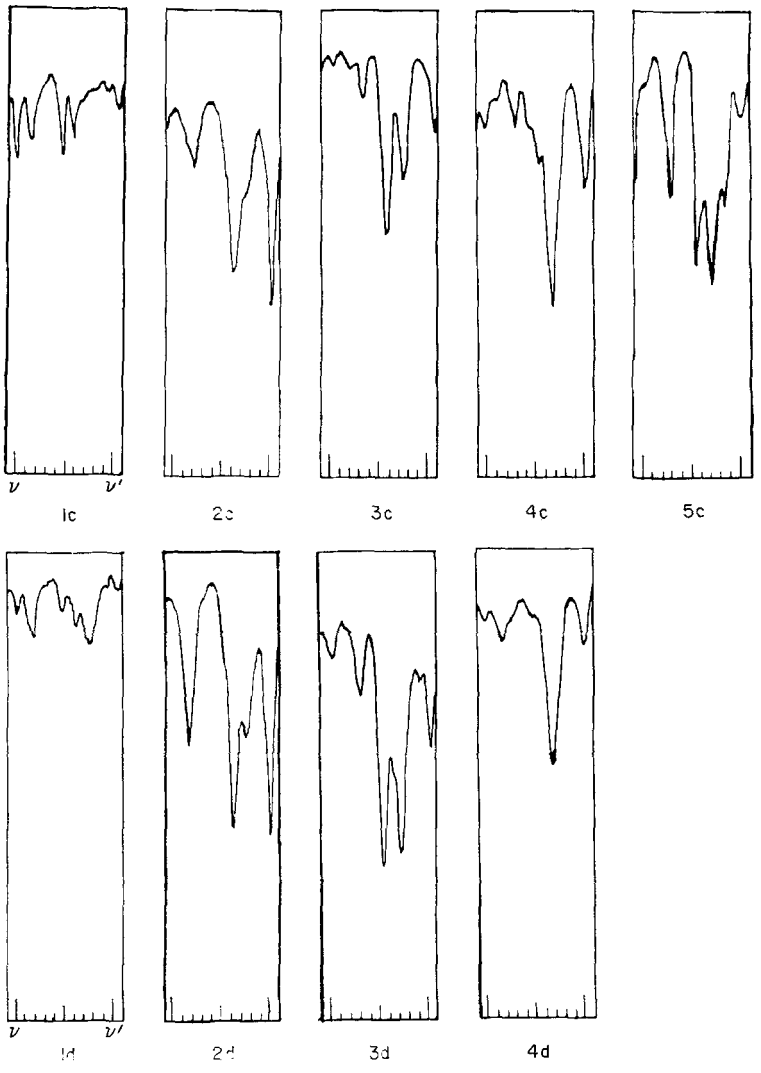

FIG. 3. IR spectra of sterols from $\nu = 900\ cm^{-1}$ to $\nu' = 800\ cm^{-1}$. Those compounds shown in the top row (c series) are saturated at C-24; those in the bottom row (d series) are Δ^{24}-sterols; 1c, cholestanol; 1d, Δ^{24}-cholestenol; 2c, Δ^{5}-cholestenol; 2d, $\Delta^{5,24}$-cholestadienol; 3c, Δ^{7}-cholestenol; 3d, $\Delta^{7,24}$-cholestadienol; 4c, $\Delta^{5,7}$-cholestadienol; 4d, $\Delta^{5,7,24}$-cholestatrienol; 5c, 4α-methyl-Δ^{7}-cholestenol.

cholestenol (1f)] does not significantly change the frequencies of major bands in this region. Also, it should be noted that major shifts in band frequencies and shapes occur either with the introduction of double bonds in Ring B or with the addition of a 4α-methyl group.

Table 5 summarizes the observed IR maxima as correlated with double bond location for the sterols examined.

DISCUSSION

Detection of the Δ^{24}-Bond by NMR

As was shown in Table 1, the C-26, C-27 methyl protons resonate at δ 0.82, 0.92 in C-24 saturated sterols. However, with introduction of a Δ^{24}-bond, a pronounced downfield shift occurs and the isopropylidene methyls at C-26, C-27 then resonate at δ 1.60, 1.68. In one case a similar assignment of the C-26, C-27, methyl protons was made by Dvornik, Kraml, and Bagli (28) in experiments in which they detected the presence of a $\Delta^{5,7,24}$-sterol as the epiperoxide in pigs treated with triparanol and AY-9944 (another inhibitor of cholesterol biosynthesis).

In addition, the C-24 olefinic proton can in some instances be resolved from olefinic protons present in the steroid nucleus.[4] Furthermore, in the spectrum of $\Delta^{5,7,24}$-cholestatrienol the C-24 proton peak was resolved into a triplet, as would be expected if spin–spin coupling interactions occurred between the C-24 proton and the two neighboring protons at C-23.[4] Also, integration of

[4] T. J. Scallen, W. J. Dean, E. D. Loughran, and B. V. Vora. Unpublished data.

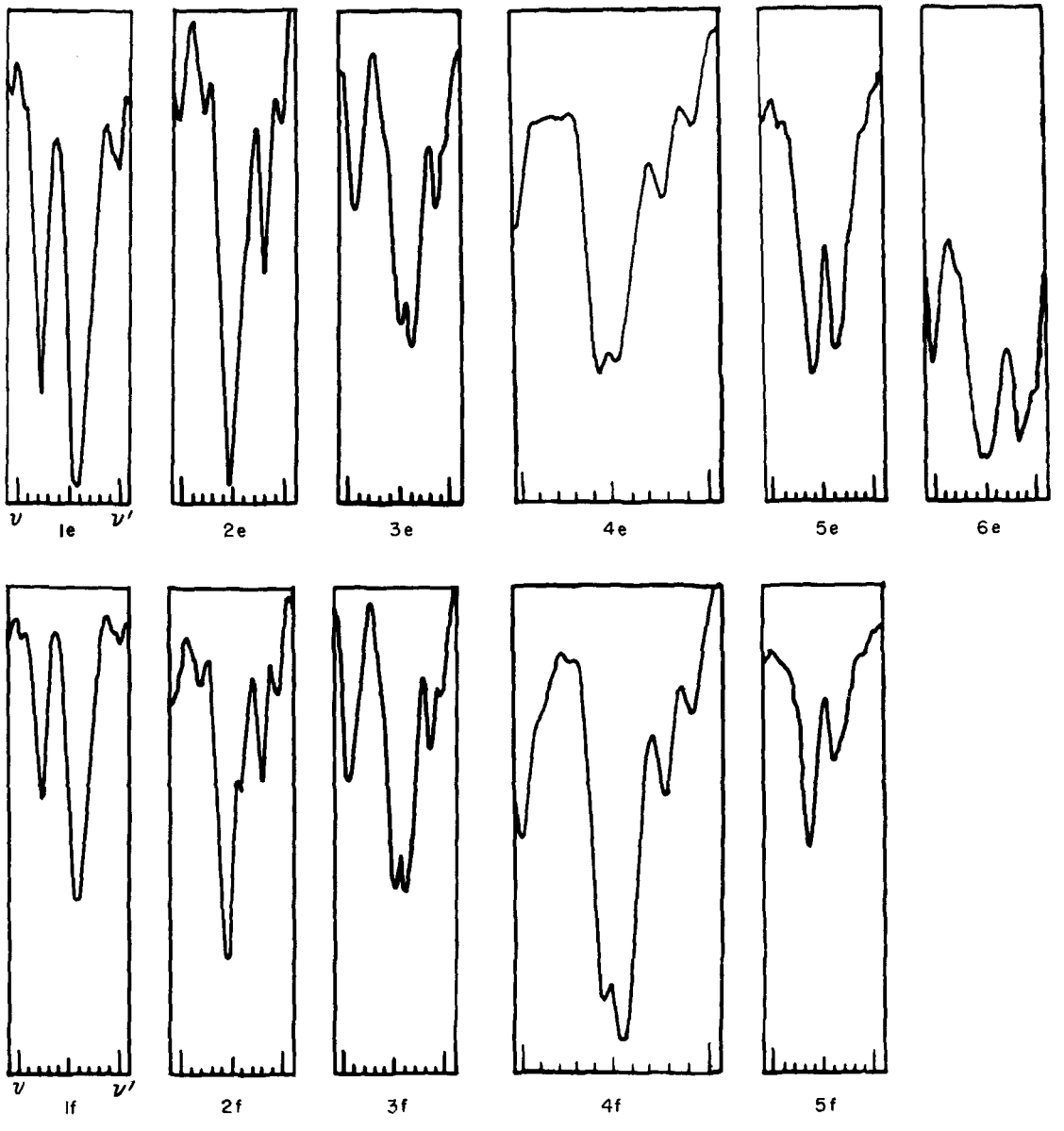

FIG. 4. IR spectra of sterols from $\nu = 1100\ cm^{-1}$ to $\nu' = 1000\ cm^{-1}$. Those compounds shown in the top row (e series) are saturated at C-24; those in the bottom row (f series) are Δ^{24}-sterols; 1e, cholestanol; 1f, Δ^{24}-cholestenol; 2e, Δ^{5}-cholestenol; 2f, $\Delta^{5,24}$-cholestadienol; 3e, Δ^{7}-cholestenol; 3f, $\Delta^{7,24}$-cholestadienol; 4e, Δ^{8}-cholestenol; 4f, $\Delta^{8,24}$-cholestadienol; 5e, $\Delta^{5,7}$-cholestadienol; 5f, $\Delta^{5,7,24}$-cholestatrienol; 6e, 4α-methyl-Δ^{7}-cholestenol.

the areas associated with olefinic protons can give the number of olefinic protons present in the molecule. When this was done for Δ^{7}-cholestenol, one olefinic proton was found to be present (at C-7), in agreement with the structure of this authentic reference material. For $\Delta^{7,24}$-cholestadienol, integration reveals two olefinic protons (one at C-7 and one at C-24), also in agreement with the structure of this sterol. If the side-chain double bond had been at Δ^{22}, Δ^{23}, or Δ^{25}, integration would have revealed three olefinic protons (one at C-7 and two for the Δ^{25}-bond); thus, Δ^{22}, Δ^{23}, and Δ^{25} are eliminated as possibilities. If a $\Delta^{20,(22)}$-system were present, a down-field shift of the C-21 methyl protons would be expected; however, inspection of Fig. 1 shows that the C-21 methyl protons in $\Delta^{7,24}$-cholestadienol have not shifted downfield and appear as a doublet, as expected for a structure with a proton present at C-20. Similar arguments would exclude a $\Delta^{17,(20)}$-system.

Thus by consideration of (*a*) the appearance of a doublet at δ 1.60, 1.68 (associated with the isopropylidene methyls at C-26, C-27); (*b*) the disappearance of the δ 0.82, 0.92 doublet (which is seen with the C-26, C-27 *gem*-dimethyls of C-24 saturated sterols); and (*c*) examination and integration of the olefinic proton re-

TABLE 5 SUMMARY OF OBSERVED INFRARED MAXIMA CORRELATED WITH DOUBLE BOND LOCATION FOR THE STEROLS EXAMINED

Group	800–900	1000–1100
		cm^{-1}
Δ^5	798–800, 824–26 (sh),* 838–839	1020, 1053–55
Δ^7	826–27, 842–45	1039–40, 1049–50
Δ^8	†	1010–11, 1023, 1046–47, 1056–57
$\Delta^{5,7}$	831–32	1038–40, 1062–64
Δ^{24}	824	‡
4α-Methyl-Δ^7	827, 845	1004, 1017, 1046, 1054
Saturated sterol skeleton‖	§	1042, 1077

* sh, shoulder.

† Tetrasubstituted double bond, therefore no specific absorption in this region.

‡ The Δ^{24}-bond has little effect upon the C–O stretching region and therefore this region is not useful for detection of this bond; however, a band present at 1365 cm^{-1} (see Fig. 2) will be decreased in relative intensity.

§ Some weak absorption is present (see Fig. 3), to judge from the cholestanol and Δ^{24}-cholestenol.

‖ As shown in the spectrum of cholestanol and Δ^{24}-cholestenol.

gion, unambiguous assignment of a Δ^{24}-bond in sterols of the cholestane type can be made. Unequivocal assignment of the biologically important Δ^{24}-bond by physical methods has not been previously reported.

Detection of the Δ^{24}-Bond by IR

Jones and Cole (22, 23) studied the IR spectra of approximately two hundred steroids. They assigned a band of medium intensity which occurred at 1368 cm^{-1} (1360–74) as being associated with the terminal *gem*-dimethyl group of the side chain in C_{27}, C_{28}, and C_{29} steroids. It was further noted that this band tended to occur above 1368 cm^{-1} in ergostane and stigmastane derivatives and below 1368 cm^{-1} in cholestane derivatives. Stokes, Fish, and Hickey (2), in reporting the isolation of $\Delta^{5,24}$-cholestadienol from chick embryos, noted the disappearance of a band near 1368 cm^{-1} that was present in a sample of cholesterol but absent from their spectrum of $\Delta^{5,24}$-cholestadienol and also of $\Delta^{8,24}$-cholestadienol. The band was also absent from the $\Delta^{5,25}$-cholestadienol spectrum; however, a strong band at 887 cm^{-1} indicated the presence of a terminal methylene group.

It is apparent from the data presented here that in most of the spectra, the band seen at 1365 cm^{-1} (1361–71) does not completely disappear but is reduced in relative intensity when a Δ^{24}-bond is introduced. However, in one case, i.e., Δ^{24}-cholestenol, the band seen at 1365 cm^{-1} in cholestanol did disappear. This suggests that the presence of unsaturation in Ring B can, in some way, contribute to weak absorption (noted as a shoulder in most of the spectra) in this region. The differences noted in our spectra from those of Stokes et al. are in most cases explicable by the higher resolution obtained with our instruments. It is apparent from our data that caution must be exercised in the assignment of the Δ^{24}-bond from IR spectra alone.

Δ^{24}-Sterols have a small band at 824 cm^{-1} associated with the C–H out-of-plane bending of the C-24 hydrogen. This band is not often useful for the detection of the Δ^{24}-bond because of the presence of more intense absorption in this region when unsaturation is present in the steroid nucleus.[5]

It has been stated (29) that a doublet at 958 and 950 cm^{-1} is typical of a $\Delta^{5,24}$-sterol. Although a strong doublet is present at 950 and 958 cm^{-1} in our spectrum of $\Delta^{5,24}$-cholestadienol, a doublet of nearly identical shape and intensity is present in our spectrum of cholesterol.

While NMR gives easily seen and clear-cut differences with introduction of a Δ^{24}-bond, a comparison of the IR spectra of Δ^7-cholestenol and $\Delta^{7,24}$-cholestadienol (17) shows that the IR differences can be rather subtle, and require high resolution as well as careful evaluation of the spectrum.

Location of Ring B Double Bonds by NMR

With very few exceptions the assumptions of Zürcher (21) concerning the additivity of chemical shifts for various substituents in the steroid nucleus have been well substantiated (20). Table 2 shows generally excellent agreement between calculation and prediction. Zymosterol ($\Delta^{8,24}$-cholestadienol) does deviate from the calculated value for the C-18 methyl protons. In this case Zürcher's calculation of the contribution for the Δ^8-bond was based upon only one Δ^8-steroid. Measurements made by Sanghvi (30) with two other Δ^8-sterols showed the C-18 methyl resonance at δ 0.595–0.608, values which lie closer to our observed value for zymosterol. A more precise estimation must depend upon further measurements with additional Δ^8-sterols.

It is important to note that a Δ^7-sterol can easily be differentiated from Δ^8-sterol, because the C-19 methyl resonance shifts from δ 0.80 (for Δ^7) to 0.93 (for Δ^8). Also, a Δ^7-sterol has one olefinic proton which can be detected at δ 5.14, whereas a Δ^8-sterol has no olefinic protons. This is important because Δ^7- and Δ^8-sterols have very similar chromatographic mobilities on silicic acid columns (18).

It might be difficult to differentiate a $\Delta^{5,7}$-sterol from a Δ^8-sterol using only the C-18 and C-19 methyl resonance frequencies; however, consideration of the olefinic proton region would show a characteristic two-

[5] However, important exceptions exist, particularly $\Delta^{8,24}$-sterols which are symmetrical about the Δ^8-bond and therefore possess specific absorption in this region that is due solely to the Δ^{24}-bond.

proton quartet[4] for the $\Delta^{5,7}$-sterol but no olefinic protons for the Δ^{8}-sterol. Of course, the UV spectrum would allow facile detection of a $\Delta^{5,7}$-sterol (31).

Location of Ring B Double Bonds by IR

Other investigators (23, 32–34) have attempted to characterize olefinic steroids by their out-of-plane C–H bending vibrations in the 800–900 cm^{-1}. Of the compounds of interest here, only Δ^{5}-cholestenol and Δ^{7}-cholestenol have been studied in this respect. Δ^{8}-Sterols, being symmetrically tetrasubstituted, give no specific absorption in this region. Fig. 3 shows that for the sterols examined, the Δ^{5}-, Δ^{7}-, and $\Delta^{5,7}$-bonds may be identified from consideration of the 800–900 $cm.^{-1}$ region.

An intense series of bands near 1000–1080 $cm.^{-1}$ have been assigned to the C–O stretching vibration of secondary alcohols (24–27) i.e., the C-3 hydroxyl group in the compounds studied here. The position of this band has been found to depend upon the stereochemistry at C-3 and C-5 (35, 36). However, the effect of unsaturation in Ring B upon this vibration has not been previously studied. It is evident from the findings described here that substituents in either Ring A or Ring B are capable of significant interaction with the C–O stretching vibration at C-3 to produce changes in band frequencies and shapes. On the other hand, the presence or absence of a Δ^{24}-bond has only a minor effect on this region; this is reasonable because of the remoteness of the Δ^{24}-bond from the C-3 hydroxyl group.

The ability to obtain structural information from small amounts of material is of key importance for biologically active compounds. The sample size for IR analysis can be 50–100 μg and still provide high resolution. The sample sizes for NMR spectra reported here ranged from 5 to 50 mg; however, by using techniques which rapidly scan a spectrum many times and average out random noise, a great increase in signal-to-noise ratio is possible. With such a device, a satisfactory spectrum was obtained for a 56 μg sample of 19-norprogesterone (20).

In conclusion, the nondestructive physical methods described here (particularly NMR) allow the unequivocal assignment of the biologically important Δ^{24}-bond in cholesterol procedures; this has not previously been possible.

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