0.02 for the other ketones. This is analogous with the results of photochemical studies, where it was found that ketones containing γ-hydrogens have a primary quantum yield less than unity[19,20]. The low quantum yield was attributed to an internal degradation of the energy in the molecule. In this connection it may be mentioned that ethylene and propylene were major products in the radiolysis of methyl *n*-propyl ketone and methyl *n*-butyl ketone, respectively.[18] This is in analogy with photolysis data and the mass spectra cracking pattern of these compounds.

(19) W. Davis, Jr., and W. A. Noyes, Jr., THIS JOURNAL, **69**, 2153 (1947).
(20) C. R. Masson, *ibid.*, **74**, 4731 (1952).

Acknowledgments.—The authors wish to thank Professors W. Albert Noyes, Jr., and E. O. Wiig, for advice and encouragement.

ROCHESTER 20, NEW YORK

[CONTRIBUTION FROM THE INSTRUMENT DIVISION, VARIAN ASSOCIATES, AND THE CHEMISTRY DEPARTMENT, MICHIGAN STATE UNIVERSITY]

Nuclear Magnetic Resonance Spectra of Steroids

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The high resolution nuclear magnetic resonance spectra of forty-seven steroids have been obtained and a number of peak positions have been measured with an accuracy of approximately 1 c.p.s. relative to the resonance of pure benzene. Empirical correlation of the data with the known steroid structures has allowed the assignment of characteristic group frequency shifts to a number of chemical functional groups. The applicability of these shifts to analysis of unknown steroids is discussed. Axial and equatorial protons in the 3-position are found to be shifted apart more than 20 c.p.s., and 18 ± 5 c.p.s. in the 11-position. Finally, the sensitivity of this method of analysis is discussed and found to be adequate to permit samples of a few milligrams to be studied.

Introduction

During the past few years an increasing number of laboratories have begun to apply the high resolution nuclear magnetic resonance (n.m.r.) technique to the solution of chemical structure problems. A general understanding of the experimental conditions which must be satisfied in order for useful spectra to be obtained is becoming more widespread. However, two of these conditions have often been believed to be more stringent than is actually the case. These are, first, the requirement of rapid random molecular motional averaging of direct dipole–dipole broadening which leads to the necessity for a liquid sample and, second, the need for a fairly high concentration of the molecular species being studied in order to achieve a useful signal-to-noise ratio. As a result, there has been a tendency to regard molecules in the molecular weight range from 300 to 500 as difficult to study *via* high resolution n.m.r. techniques, and above 500 as virtually impossible. However, the early studies from which such conclusions were drawn were usually concerned with long chain hydrocarbons which are viscous liquids, or polynuclear aromatic compounds which are too high melting for study without recently developed high temperature apparatus[3] and too insoluble in solvents with low proton backgrounds. Recent work[4] has shown that diluting viscous liquids with several volumes of a proton-free, low-viscosity solvent does, indeed, often permit enough molecular motional averaging to occur, even at room temperature, to give acceptably narrow resonance lines. The same results might be expected to hold for dilute solutions of normally solid substances of comparable molecular weight. The present study was carried out to find suitable experimental conditions under which sufficiently well resolved spectra of steroids could be obtained to permit assignments to be made, to assign the resonance peaks wherever possible and to correlate the assignments with the structural features of the steroid molecules.

For reasons described later it was desirable to obtain all of the spectra in the same solvent. Chloroform possesses the necessary solvent properties and its single sharp resonance does not interfere with the resonances of any of the steroids employed in this study, but even the spectrophotometric grade offered commercially gives a whole spectrum of impurity peaks which are comparable in size to many of the signals from the solute. If suitable purification techniques were employed, chloroform could be used successfully as a solvent. It is easier, however, to use deuterated chloroform which is commercially available and which is completely free of background interference.

The optimum concentration for this work lies in the vicinity of a 0.5 *M* solution. Such a solution is obtained from 50 to 75 mg. of steroid (depending on the molecular weight) dissolved in 0.4 cc. of $CDCl_3$, a typical cell volume. An encouragingly large number of steroids are soluble to this extent and if acetate derivatives are prepared most others can be made to fall into this category. Higher concentrations are likely to lead to excessive viscosity with consequent line broadening and are often too demanding on the amount of pure compound available. Lower concentrations make difficult the measurement of the positions of certain broad, low peaks in the spectrum which are among the more important chemical shift measurements which may be desired; however, this loss of signal-to-noise ratio is offset by several compensating factors, among which might be mentioned diminished viscosity

(1) Instrument Division, Varian Associates, Palo Alto, California.
(2) Michigan State University, East Lansing, Michigan.
(3) J. N. Shoolery and J. D. Roberts, *Rev. Sci. Instr.*, **28**, 61 (1957).
(4) Unpublished studies, Varian Associates.

broadening, closer approach to uniformity of the sample volume magnetic susceptibility and the smaller quantity of sample required.

Experimental

All spectra were obtained at 40 megacycles/second in a magnetic field of approximately 9400 gauss. The compounds were studied in dilute solution in deuterated chloroform, concentrations ranging from approximately 0.1 to 0.5 *M* depending on the amount of each steroid available or, in a few cases, upon the solubility. The zero of reference in each spectrum was taken as the resonance position of pure benzene contained in a precision external annular cell.[5] The shift values of the sharp peaks in the spectrum of each steroid were determined by the audiofrequency side band method[6] in cycles per second and could be reproduced to within 1 c.p.s. The sign of the shift is chosen to be positive when the resonance falls at a higher applied field than the reference. With this definition, the frequency of a resonance unsplit by spin–spin coupling is proportional to the total shielding, relative to benzene, at the proton group being studied. For comparison with work done at other field strengths it will often be desirable to convert the position of a peak (or center of a spin–spin multiplet) to field-independent units. The position of any point in the spectrum in the generally accepted dimensionless units, δ, can be determined by dividing by 40 the frequency obtained from linear interpolation between measured peaks. This corresponds to the definition $\delta = 10^6 \times (H - H_{ref})/H_{ref}$.

The deuterated chloroform[7] used as solvent was found by n.m.r. assay to be 99.5% $CDCl_3$. No additional hydrogen-containing impurities could be detected in a blank run. The steroids for this study were supplied either as experimental samples through the kind coöperation of the research departments of Merck and Co., Inc., the Schering Corp., and the Upjohn Co., or from the collection of steroids belonging to one of us (MTR). The latter group had been recrystallized and the melting points were in good agreement with values in the literature. Purity is not critical in high resolution n.m.r. work as far as assignment of peaks is concerned, since the area under each peak is proportional to the number of protons in that particular chemical environment. None of the spectra gave any indication of peaks which could not be assigned on the basis of a single steroid species. Table I lists the steroids studied and the shifts in c.p.s. of the angular methyl proton resonances.

The spectrometer employed for these measurements was a Varian Associates V-4300-B High Resolution NMR Spectrometer with associated 12″ Magnet System equipped with a V-K3506 Flux Stabilizer. Samples were placed in precision ground Pyrex tubes with 5 mm. o.d. and 4 mm. i.d. and rotated at several hundred r.p.m. by a small air turbine during the recording of the spectra. Audio-frequency side bands for calibration purposes were generated with a Hewlett-Packard 200-CD Audio Oscillator and measured with a Hewlett-Packard 521-C Frequency Counter.

With careful attention to experimental technique it is possible to achieve sufficient sensitivity to permit the use of steroid samples in the range of only a few milligrams. Among the most important factors are: (1) regulation of input a.c. line voltage to the spectrometer to $\pm 0.1\%$[8]; (2) adjustment of coupling between transmitter and receiver coils of the n.m.r. sensing head to the minimum possible value consistent with obtaining the desired absorption mode of the resonance signals; (3) use of thin-walled cells of high filling-factor; (4) use of minimum amount of solvent—just sufficient to fill the cell to receiver coil level; (5) employment of sufficiently slow sweep rate (1 c.p.s.) and a suitable receiver time constant (approximately one second). Signals from a 0.02 *N* concentration of hydrogen nuclei giving a single resonance line can be detected with a two-to-one signal-to-noise ratio. For example, the $CDCl_3$ solvent (99.5% deuterated) still retains a 0.06 *M* concentration of $CHCl_3$, which gives a signal-to-noise ratio of six to one. According to these figures, one would predict that a steroid of molecular weight 400 would give detectable signals at a concentration of 8 mg./ml., or 3.2 mg. in the typical 0.4 ml. cell volume. From the following sections of this paper it will be noted, however, that the majority of significant information is associated with the chemical shifts of the angular methyl peaks. If one is willing to settle for this restriction, measuring only methyl peaks, it should be possible to divide the sample requirement by three, giving a figure slightly in excess of 1 mg.

A slight modification of the sample cell of the V-4300B Spectrometer, to be described elsewhere, was effected which

TABLE I

ANGULAR METHYL PROTON RESONANCE SHIFTS IN STEROIDS

Steroid[a]	C_{18}-shift, c.p.s.	C_{19}-shift, c.p.s.
$\Delta^{5(6)}$-Cholestene-3β-acetate	228[b]	215
Δ^{4}-Androstene-17β-ol-3-one	224	208
Δ^{4}-Androstene-17β-ol-17α-methyl-3-one	220	208
19-Nortestosterone	223	..
Δ^{4}-Pregnene-9α-fluoro-11β,21-diol-3,20-dione-21-acetate	218	193
Δ^{4}-Pregnene-21-ol-3,11,20-trione	229	200
Δ^{4}-Pregnene-3,20-dione-21-acetate	228	208
$\Delta^{5(6)}$-Pregnene-3β-ol-20-one	230	215
Δ^{4}-Pregnene-3,20-dione	232	210
Allopregnane-3,11,20-trione	233	208
Pregnane-3,11,20-trione	231	207
Pregnane-3,20-dione	230	215
Allopregnane-3,20-dione	231	215
Δ^{4}-Pregnene-11β-ol-3,20-dione	220	197
Δ^{4}-Pregnene-11α-ol-3,20-dione	228	204
Δ^{4}-Pregnene-11α-acetoxy-3,20-dione	228	206
$\Delta^{5(6),16}$-Pregnadiene-3β-ol-20-one	220	214
$\Delta^{5(6)}$-Pregnene-3β-methoxy-20-one	231	216
$\Delta^{5(6)}$-Pregnene-3β,21-diacetoxy-20-one	229	215
$\Delta^{5(6)}$-Pregnene-3β,21-diol-20-one-21-acetate	229	215
$\Delta^{5(6),16}$-Pregnadiene-3β-acetate-20-one	219	214
Allopregnane-3,20-dione-21-acetate	229	215
Δ^{4}-Pregnene-3,11,20-trione	231	200
$\Delta^{5(6)}$-Androstene-3β-ol-17-one	220	215
$\Delta^{5(6)}$-Androstene-3β-acetate-17-one	220	214
Allopregnane-11α-ol-3,20-dione	229	210
Stigmasteryl acetate	228	216
$\Delta^{5(6)}$-Pregnene-3β-acetate-20-one	231	215
Δ^{4}-Androstene-3,11,17-trione	222	199
Estrone	222	..
Androstane-3β-ol-17-one	222	221
5,6-Dihydroergosteryl acetate	234	223
Δ^{4}-Pregnene-17α-ol-3,20-dione	227	207
Pregnane-4-chloro-17α-ol-3,11,20-trione	234	208
Δ^{4}-Pregnene-11β,21-diol-3,20-dione	218	197
Pregnane-11α-ol-3,20-dione	231	211
Androstane-3α-ol-17-one	223	221
Etiocholane-3α-ol-17-one	223	219
$\Delta^{4,17(20)}$-Pregnadiene-11β,21-diol-3-one	209	197
$\Delta^{4,9(11)}$-Pregnadiene-17α,21-diol-3,20-dione	230	203
Δ^{1}-Allopregnene-17α,21-diol-3,11,20-trione-21-acetate	230	205
Pregnane-3α-acetoxy-11,20-dione	233	210
Pregnane-3α-ol-11,20-dione	233	212
$\Delta^{4,9(11)}$-Pregnadiene-17α,21-diol-3,20-dione-21-acetate	232	202
Ergosterol	231	218
Δ^{5}-Cholestene-3-one	228	209
$\Delta^{5(6),7}$-Cholestadiene-3β-acetate	231	219

[a] Solutions in $CDCl_3$. [b] Relative to benzene in external annulus.

(5) Wilmad Glass Co., Landisville, New Jersey.

(6) J. T. Arnold and M. E. Packard, *J. Chem. Phys.*, **19**, 1608 (1951).

(7) Merck, Ltd., Montreal, Canada.

(8) This is within the capability of commercially available a.c. regulators. Line voltage was regulated only to the V-4300B and not to the V-2100 magnet supply.

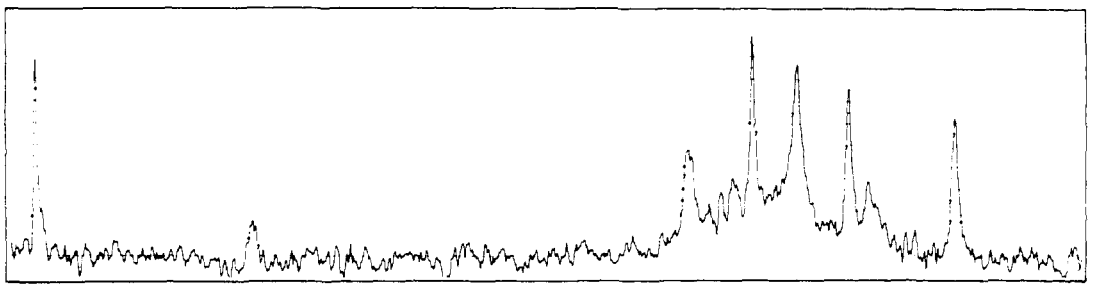

Fig. 1.—Nuclear magnetic resonance spectrum of 1.35 mg. of 11-ketoprogesterone as 0.02 *M* solution in $CDCl_3$.

permitted the volume to be reduced to 0.2 ml. without loss of sensitivity. A sample of 11-ketoprogesterone weighing 1.35 mg. was dissolved in this volume of $CDCl_3$, giving a 0.02 *M* solution. The spectrum is shown in Fig. 1. This spectrum shows that detectable methyl resonances could have been obtained from 450 μg. of this steroid species.[9]

Discussion

General Features of N.M.R. Spectra of Steroids.—The compounds which have been studied so far have yielded spectra with peaks falling as low as 57 c.p.s. ($\delta = -1.42$) below the benzene reference and as high as 234 c.p.s. ($\delta = +5.85$) above this point. Since the measurements are made with the reference substance and the steroid solution physically separated and coaxially located, the protons in the reference sample and those in the steroid molecules find themselves in media of different bulk magnetic susceptibilities. Although both benzene and the steroid solutions are diamagnetic, the volume susceptibility of the chloroform is lower (more diamagnetic) than that of benzene, resulting in a shift of the resonance of the steroid to lower applied fields in chloroform solution than in a solution of the same susceptibility as benzene. It is therefore important to compare such spectra only when the shift measurements have been made in the prescribed manner and in solutions of approximately the same concentration. Some experiments were performed which showed that the most concentrated steroid solutions, which were approximately 1 *M*, showed a shift of about 2 c.p.s. of all peaks to higher values since the susceptibility was slightly higher (less diamagnetic) than the dilute solutions containing more $CDCl_3$. This 2 c.p.s. uncertainty is less than the uncertainties in most of the useful correlations derived for steroid spectra and can be estimated and taken into account with even a very crude measurement of the concentration.

One might suggest that the data could be corrected for the bulk susceptibility differences, but this would only be justified if (1) the susceptibilities were measured for each solution and (2) the correction for cylindrical samples is correctly given by the formula Δf(c.p.s.) $= (4 \times 10^7)(2\pi/3)(\kappa_{ref} - \kappa_{soln})$ where Δf should be added to the observed shifts to correct for the difference in the volume susceptibilities, κ, of reference and solution. However, recent work[10,11] has demonstrated deviations from the factor $2\pi/3$ which have been attributed to magnetic anisotropy of the solvent molecules induced by the solute molecules.

Another approach, also demonstrated[12] to be subject to uncertainties, would be to add a trace of benzene or other reference compound to the solution. Although the bulk susceptibility in the vicinity of both reference and sample molecules should then be the same, the danger of concentration dependent shifts due to weak molecular interactions or permanent magnetic anisotropy of the solvent molecules is always present. This procedure would also contaminate the $CDCl_3$ with the reference compound. The $CDCl_3$ usually can be recovered without noticeable exchange of hydrogen with the steroid molecules.

Spectra obtained for two steroid compounds in both CCl_4 and CS_2 solutions showed almost no deviation from the values obtained for $CDCl_3$ solutions, but a solution in deuterated acetone showed an increase in the shifts of 25 c.p.s., in accord with the measured volume susceptibilities of these solvents. Direct comparison of spectra taken in CCl_4 or CS_2 with the results of this study should, however, be regarded with caution.

Two ranges of relaxation times seem to be associated with the proton resonances in steroids, since some of the lines are several times as broad as others. The broad lines are attributed to protons attached directly to a carbon atom of the perhydro-1,2-cyclopentenophenanthrene skeleton, since complete averaging of the direct magnetic dipole–dipole interactions with other skeletal protons requires such rapid random reorientation of the entire 4-ring system that it is apparently not possible at room temperature, even in the dilute solutions studied here. The skeletal protons are seldom more than one carbon atom removed from at least one other such proton, and this results in splitting the already broad resonance into a number of spin–spin components, due to the $\vec{I}\cdot\vec{I}$ interaction of the proton spins with one another through the intervening bonds. The chemical shifts of skeletal protons relative to one another are often not large compared to the strength of the spin coupling between them; consequently, a number of unresolved spin–spin components usually are observed. An interpretation of these would, in general, require the application of second-order perturbation theory or even a solution of a fairly complex secular equa-

(9) By use of a spectrometer frequency of 60 cm. (*e.g.*, Varian Associates Model V-4300-C spectrometer) the sample requirement may be reduced by a factor of about $^2/_3$; also chemical shifts are increased by a factor of $^3/_2$ at the higher frequency thus improving the ability to distinguish between different chemical groups.

(10) A. A. Bothner-By and R. E. Glick, *J. Chem. Phys.*, **26**, 1647 (1957).

(11) A. A. Bothner-By and C. Naar-Colin, Presented at 132nd National Meeting, A.C.S., New York, 1957.

(12) A. A. Bothner-By and R. E. Glick, *J. Chem. Phys.*, **26**, 1651 (1957).

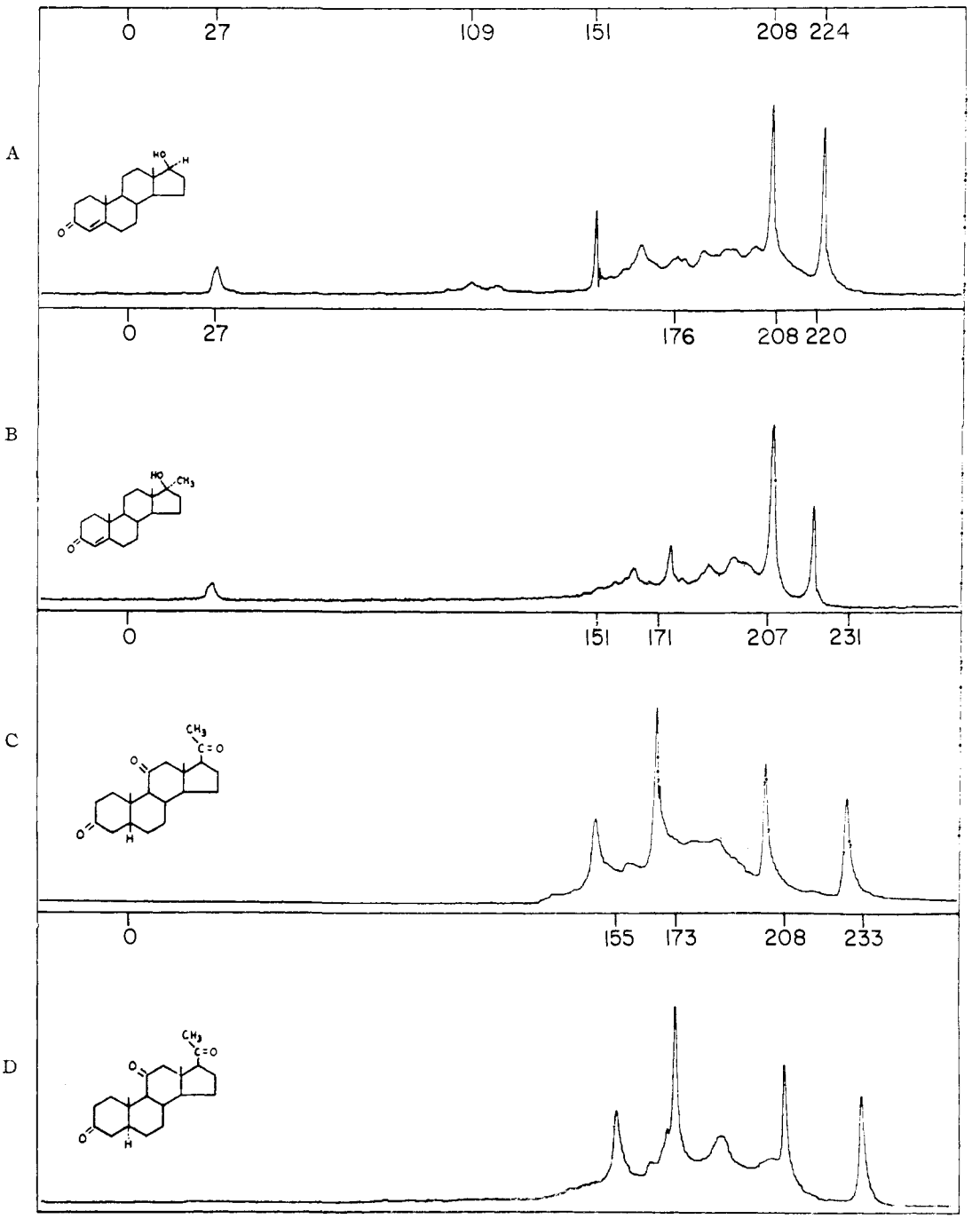

Fig. 2.—Nuclear magnetic resonance spectra in $CDCl_3$ solution: A, testosterone (Δ^4-androstene-17β-ol-3-one); B, 17α-methyltestosterone; C, pregnane-3,11,20-trione; D, allopregnane-3,11,20-trione.

tion, due to the possibility of several groups of protons being strongly coupled. Finally, it is quite likely that skeletal protons separated by too many bonds to permit observable spin coupling may still accidentally give resonances which fall directly atop one another, and this would make a detailed interpretation still more difficult.

The consequence of the strong spin–spin couplings and small chemical shifts associated with the skeletal proton resonances is that these resonances give a broad "hump" of complex structure, underlying the sharper peaks due to methyl protons, in the region of the spectrum from approximately δ = +3.5 to +5.8 relative to the benzene resonance. Although not subject to detailed interpretation, this background absorption serves as an extremely characteristic "fingerprint." Disturbing even a single proton can produce a marked effect on the "fingerprint," as in the case of the region between 180 and 208 c.p.s. in testosterone and 17α-methyltestosterone,[13] shown in Figs. 2A and 2B, where the

(13) For a discussion of the stereochemistry of the steroids, the reader is referred to Chapter X of "Natural Products Related to Phenanthrene," Third Edition, by L. F. Fieser and M. Fieser, Reinhold Publ. Corp., New York, N. Y., 1949.

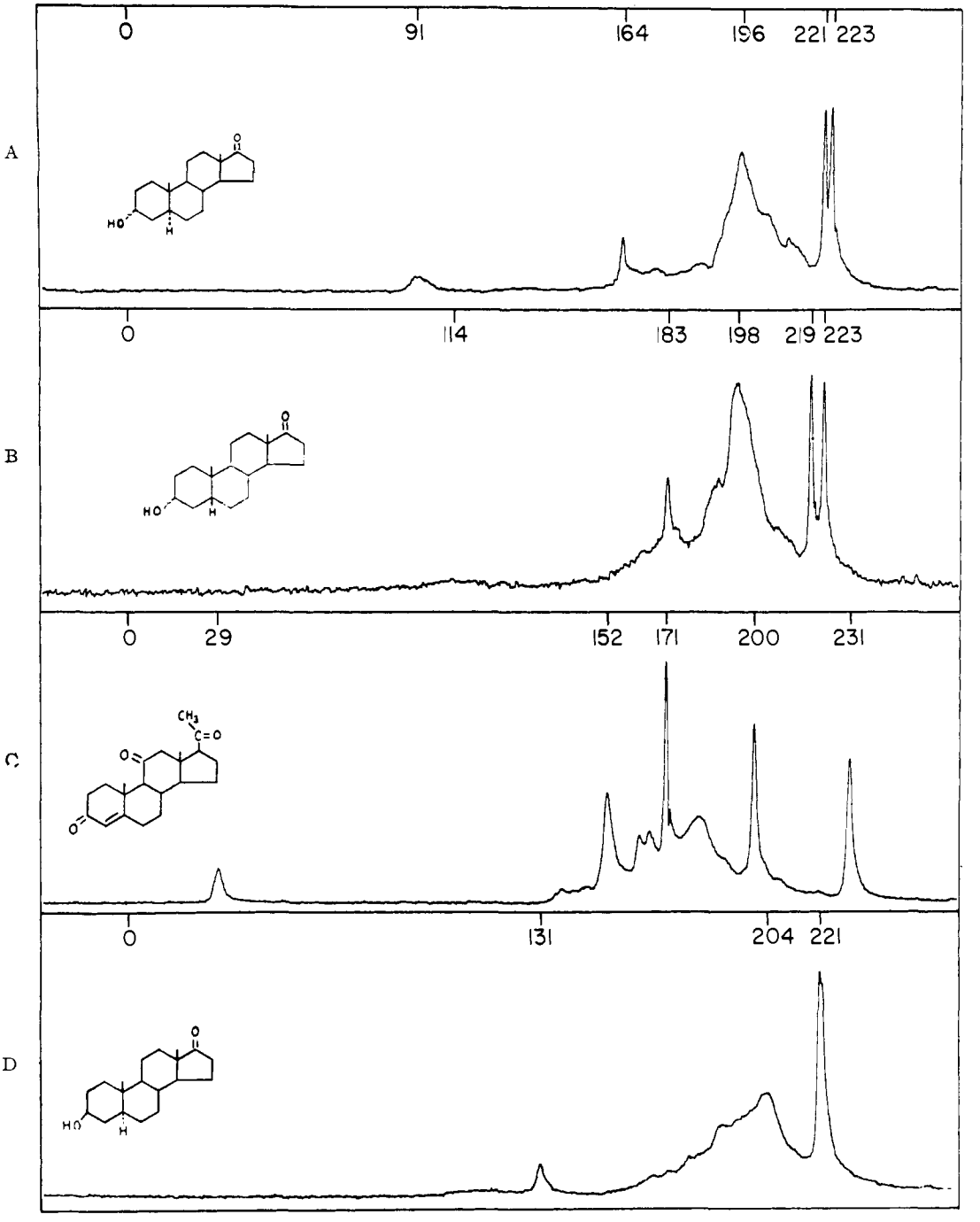

Fig. 3.—Nuclear magnetic resonance spectra in $CDCl_3$ solution: A, androsterone (androstane-3α-ol-17-one); B, 5-isoandrosterone (etiocholane-3α-ol-17-one); C, 11-ketoprogesterone (Δ^4-pregnene-3,11,20-trione); D, epiandrosterone (androstane-3β-ol-17-one).

proton on C_{17} has been replaced by a methyl group in the latter compound. Changes in configuration can also strikingly alter the appearance of the spectrum in this region as shown by steroid pairs of the pregnane and allopregnane type in Figs. 2C and 2D, or in the case of androsterone and 5-isoandrosterone, shown in Figs. 3A and 3B.

The narrow lines are attributed to groups which, through attachment to the steroid framework *via* a single bond, possess an additional rotational degree of freedom. Methyl groups in particular fall into this category and due to the equivalence of the three protons in the group they give resonances of very large amplitude, since the total intensity corresponding to all three protons is squeezed into a narrow line. The positions of these lines can be measured rather easily, with a reproducibility better than one cycle per second. Consequently, they serve as a fairly sensitive indication of the various structural perturbations which can be encountered, such as the presence of double bonds, hydroxyl, halogen, keto-groups, and others. The exact posi-

tions of the lines depend upon a sum of diamagnetic and paramagnetic shifts, the former being associated principally with the electron density around the protons in the methyl group, and the latter being dependent upon the departure from spherical symmetry of those same electron clouds. Other more remote electrons in the molecule can also contribute if their dynamic interaction with the magnetic field is of a type which permits magnetic moments to arise when averaged over all molecular orientations. This is illustrated by the behavior of π-electrons in benzene rings,[14] which results in relatively large shifts, not only in the molecule containing the benzene ring but also in neighboring molecules.

Shifts of the angular methyl groups, C_{18} and C_{19}, in the steroids appear to vary over a range of about 25 c.p.s. for the C_{18} protons and 28 c.p.s. for the C_{19} protons. The two resonances were observed with a separation as large as 31 c.p.s. in 11-ketoprogesterone, Fig. 3C, and smaller than 1 c.p.s. (overlapping) in epiandrosterone, Fig. 3D. The resonances appear to tend toward a maximum shift to high field as the structure of the steroid in the vicinity of the methyl group approaches an unperturbed state satisfied by the following requirements: (1) that there be no double bonds present, and (2) that the number of substituent groups in the molecule near the methyl group under consideration shall be a minimum. Under these limiting conditions the C_{18} and C_{19} protons are separated by about 15 c.p.s. The separation of the centers of the ranges over which the C_{18} and C_{19} resonances are found is 17 c.p.s. These observations suggest that the angular methyl resonances in the parent perhydrodimethylcyclopentenophenanthrene hydrocarbon would be separated by about 15–17 c.p.s. and that the separation can be increased or decreased by appropriate structural modifications in the vicinity of the C_{19} and C_{18} methyl groups, respectively.

In addition to methyl groups, hydroxyl protons and methylene groups located in freely rotating side chains have been found to give sharp resonance lines, as expected.

Specific Features of Steroid N.M.R. Spectra.—A large number of n.m.r. chemical shifts are correlated with certain steroid structural features in this section.

Double Bonds.—Protons attached to carbon atoms which are forming a double bond experience the smallest magnetic shielding of any protons in the steroids studied so far. The peak positions range from -54 to $+54$ c.p.s.[15] Table II summarizes the observations. Peak assignments were straightforward except in the case of the proton on C_7 in 5,6-dihydroergosterol acetate, Fig. 4A. This compound has three protons attached to doubly bonded carbon atoms at C_7, C_{22} and C_{23}, and shows an unresolved line about 8 c.p.s. wide extending from 46 to 54 c.p.s. Stigmasteryl acetate, Fig. 4B, exhibits a double line extending from 48 to 57 c.p.s. which is assigned to the C_{22} and C_{23} protons, and a line at 42 c.p.s. assigned to the proton on C_6. These assignments suggest that the C_7 proton in 5,6-dihydroergosterol acetate probably lies on the low side of the 46–54 c.p.s. range, and its position has been assigned as 47 to 48 c.p.s.

TABLE II

PROTON RESONANCE SHIFTS FOR PROTONS ON DOUBLY-BONDED CARBON ATOMS

Position of double bond	Proton resonance obsd.	Shift,[15] c.p.s.
Δ^1-3 keto	C_1	-54^a
	C_2	19^a
Δ^{16}-20 keto	C_{16}	$-13(2)^b$
Δ^4-3 keto	C_4	23(1); 24(1); 26(2); 27(4); 28(3); 29(3); 30(1)[c]
$\Delta^{5,7}$	C_6, C_7	38(2)
$\Delta^{9(11)}$	C_{11}	39
Δ^5	C_6	40(2); 41(1); 42(5); 43(4)
$\Delta^{17(20)}$	C_{20}	44^d
Δ^7	C_7	47 to 48[e]
Δ^{22}	C_{22}, C_{23}	48 to 52(3)[e]

[a] Average of doublet line positions due to spin coupling between protons on C_1 and C_2. [b] Number in parentheses indicates the number of different compounds observed. [c] Concentrated solution. Susceptibility correction of -2 c.p.s. should be applied. [d] Shows triplet fine structure due to CH_2 group at C_{21}. Compound is $\Delta^{4,17(20)}$-pregnadiene-11β, 21-diol-3-one. [e] See text for a discussion of peak assignments.

The low values of -54 and -13 c.p.s. observed for the C_1 proton in 17α,21-dihydroxy-Δ^1-allopregnene-3,11,20-trione-21-acetate and for the C_{16} proton in 16-dehydropregnenolone, Fig. 4C, seem to be associated with the location of these protons on the end carbon atom of the conjugated system. Protons on middle carbon atoms all fall on the high field side of benzene. Ring size and steric effects could also be important. Conjugation of a Δ^4-double bond with a 3-keto group seems to account for about 15 c.p.s. shift to lower fields, since the resonance in fifteen different compounds is found at an average value of 27 c.p.s., compared to an average of 42 c.p.s. for twelve different $\Delta^{5(6)}$-unconjugated steroids.

Integrated intensities in this region of the spectrum can be very useful if the steroid is sufficiently soluble to avoid signal-to-noise problems and if enough sample is available to take advantage of such solubility. For a given sample run under a given set of experimental conditions, the total area under all peaks assignable to the protons in a particular chemical environment depends only on the number of such protons and their relaxation times. Since the latter are difficult to measure and correct for, the usual practice is to run the spectrometer at very low r.f. power levels, or to operate at more than one power level and extrapolate to zero r.f. power. At sufficiently low power levels[16] the terms dependent upon the relaxation times drop out, and the ratios of areas under various regions of the spectrum assigned to protons in particular chemical environments should take on values which are the ratios of integers, in particular, the integers representing the

(14) H. J. Bernstein, W. G. Schneider and J. A. Pople, *Proc. Roy. Soc. (London)*, **A236**, 515 (1956).

(15) All measurements in $CDCl_3$ solution relative to external annular benzene reference. Hereafter all peak positions will be given in c.p.s. with this system of referencing unless otherwise stated.

(16) Determined by the condition $(\gamma H_1)^2 T_1 T_2 << 1$, where γ is the magnetogyric ratio, H_1 is the radiofrequency rotating magnetic field strength, and T_1 and T_2 are the longitudinal and transverse relaxation times, respectively.

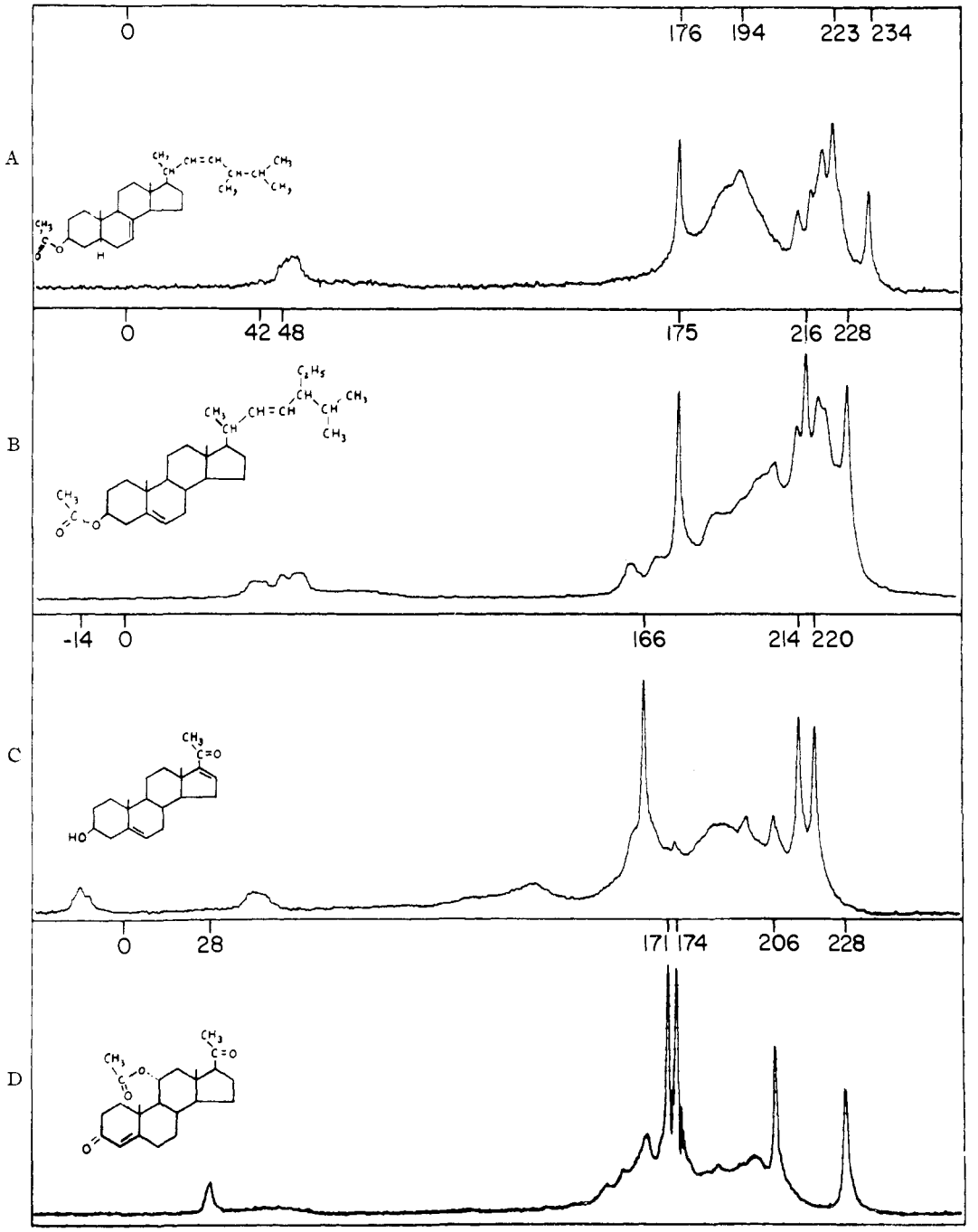

Fig. 4.—Nuclear magnetic resonance spectra in $CDCl_3$ solution: A, 5,6-dihydroergosteryl acetate; B, stigmasteryl acetate; C, 16-dehydropregnenolone ($\Delta^{5(6),16}$-pregnadiene-3β-ol-20-one); D, 11α-acetoxyprogesterone.

number of such protons in the chemical formula of the steroid. It is usually possible to determine the area corresponding to one proton by measuring the area under a methyl group, such as C_{18}, and dividing by three. The number of protons on unsaturated carbon atoms can then be estimated by comparison of areas. Due to experimental difficulties, an exact integer is seldom obtained, even for pure compounds, but it is usually possible to decide whether there are one, two or three such protons. This can be of great value in determining whether a suspected double bond is common to two rings (no protons), involves one ring juncture (one proton), or is isolated from ring junctures (2 protons).

The only interfering group found in the steroids studied in this work is the proton attached to the same skeletal carbon atom as an acetoxy group. In 11α-acetoxyprogesterone, Fig. 4D, a broad, low peak appears at 48 c.p.s. which can only be attributed to this source. Steroids with 3β-acetoxy groups show a similar peak in the vicinity of 75 c.p.s., and, for reasons discussed further on, we expect some 3α-acetoxysteroids to show such a peak around 55 c.p.s. Due to the breadth of these inter-

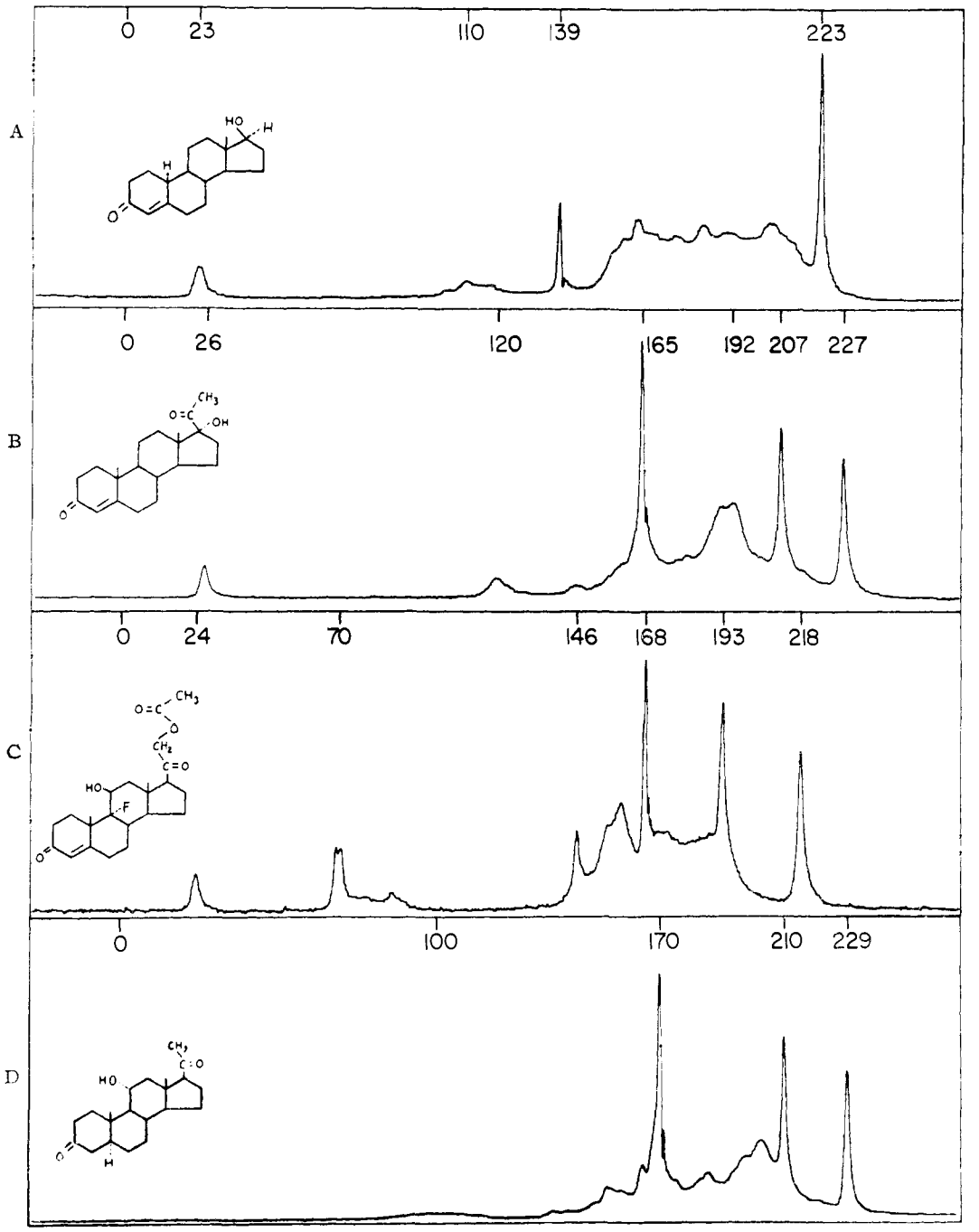

Fig. 5.—Nuclear magnetic resonance spectra in $CDCl_3$ solution: A, 19-nortestosterone; B, 17α-hydroxyprogesterone (Δ^4-pregnene-17α-ol-3,20-dione); C, 9α-fluorocorticosterone acetate (Δ^4-pregnene-9α-fluoro-11β,21-diol-3,20-dione-21-acetate); D, 11α-hydroxyallopregnane-3,20-dione (allopregnane-11α-ol-3,20-dione).

fering peaks, they appear as a broad base beneath the =CH resonance and can be recognized and taken into account in interpreting the integrated intensity in this region.

Hydroxyl Groups.—The peak corresponding to a hydroxyl group in a steroid can vary considerably in width and position, depending upon the temperature and concentration. Hydrogen bonding has been shown to lead to a large shift in the resonance position of the hydroxyl group of ethanol.[17-19] This shift is in the direction of decreasing shielding with increasing hydrogen bonding. Only in quite concentrated steroid solutions (>1 M) is sufficient hydrogen bonding encountered to shift the hydroxyl peak below the complex skeletal proton resonance region. The 17β-hydroxyl resonances in testosterone, Fig. 2A, 17α-methyltestosterone, Fig. 2B, and 19-nortestosterone, Fig. 5A, can be identified readily by their temperature and concentration dependence

(17) J. T. Arnold and M. E. Packard, *J. Chem. Phys.*, **19**, 1608 (1951).

(18) A. D. Cohen and C. Reid, *ibid.*, **25**, 790 (1956).

(19) E. D. Becker, U. Liddel and J. N. Shoolery, *J. Mol. Spec.*, **2**, 1 (1958).

to be the sharp peaks at 151, 176 and 139 c.p.s., respectively, as can the 17α-hydroxyl resonance at 120 c.p.s. in 17α-hydroxyprogesterone, Fig. 5B. Similarly, fairly sharp 3-hydroxyl resonances of androsterone, Fig. 3A, epiandrosterone, Fig. 3D, and 5-isoandrosterone, Fig. 3B, can be identified at 164, 131 and 183 c.p.s., respectively. The range of shift values observed indicates that considerable variation occurs in the degree of association taking place *via* the hydroxyl proton. Part of this variation may be due to differences in the concentrations which gave optimum spectra for each steroid, but the range of shift values is large enough to suggest that they can reflect to some extent the hydrogen-bond forming abilities of these steroids relative to one another. Further studies in which the shifts for different steroids at identical concentrations were compared would be interesting.

Several 11-hydroxysteroids were studied and in only one case can a peak be assigned unambiguously to an 11-hydroxy proton. The resonance from such a proton appears generally to fall above 170 c.p.s. and to be somewhat broadened, so that it does not stand out from the background absorption due to the skeletal protons in this region. The high shift value suggests that the 11-hydroxy group is unfavorably placed with respect to ease of intermolecular association. The one exception noted, 9α-fluorocorticosterone acetate, Fig. 5C, with the hydroxyl peak at 146 c.p.s., may be associating *via* the 9α-fluorine.

The generally lower shielding values for 3-hydroxy and 17-hydroxysteroids relative to those for 11-hydroxysteroids suggest that in solution the steroid molecules can more readily approach one another with hydroxyl groups properly oriented for formation of a hydrogen bond if the hydroxyl groups are located near the end of the molecule than if they are near the center. Since hydrogen bond formation was not observed to be strong enough to shift the hydroxyl resonance into the observable region (below 170 c.p.s.) in the case of 11α-hydroxypregnane-3-20-dione, Fig. 5D, the general shape of the steroid molecule as well as the presence of the methyl groups C_{18} and C_{19}, which will sterically hinder hydrogen bond formation *via* the 11β-hydroxy group, is indicated as a factor to be considered in explaining the high 11-hydroxy proton shielding.

Steroids with primary hydroxyl groups in side chains, such as 11-dehydrocorticosterone, Fig. 6A, appear to be strongly involved in hydrogen bonding, the temperature dependent hydroxyl peak being found at 54 c.p.s.[20]

Dissociable protons in an aggregation of molecules may be continuously undergoing exchange among a number of possible sites with different shielding; furthermore, the shielding of a hydroxyl proton can change abruptly if the group suddenly becomes involved in intermolecular association or terminates such an association. Gutowsky and Holm[21] have treated this problem and shown that if the exchange of protons occurs in an average time which is short compared to the frequency separation of the resonances in the sites between which the exchange is taking place, a single sharp resonance is observed whose position depends upon the time spent at each of the various sites. If the exchange is slow, individual sharp resonances are observed corresponding to the protons at each site; and if the exchange rate lies in the range from about $^1/_{10}$ to 10 times the frequency shift associated with exchange, *broadening* of the resonance occurs. Since the rates of the two processes involving hydroxyl protons, *i.e.*, exchange and hydrogen bond formation or rupture, can vary greatly depending on the presence or absence of trace amounts of hydrogen ions, and also depend upon molecular shape and concentration, it is not surprising that a wide variation in the line width of the hydroxyl resonance is encountered in going from steroid to steroid, or even from one sample to another of a specific compound.

Methoxy Groups.—Only one compound containing this group has been studied, namely, pregnenolone-3-methyl ether, Fig. 6B. As in many other compounds, the oxygen appears to exert a strong electron withdrawing effect, with resulting decrease in shielding of the protons in the attached methyl group. The shift value of 122 c.p.s. does not fall near any other sharp peak of an amplitude corresponding to three protons, and therefore this group can be identified unambiguously in the steroids of the type studied so far. Derivatives with methyl groups bonded to nitrogen would represent the most likely possibility of conflict.

20-Keto-C_{21} Steroids.—The shift of the C_{21} methyl group in 17 different 20-ketosteroids has been measured and found to fall at 171 $\pm$ 1 c.p.s. with two exceptions. The actual distribution of shifts (number of compounds studied in parentheses) is: 171 (10); 172 (2); 170 (3); 166 (1); 165 (1). The exceptions, 16-dehydropregnenolone, Fig. 4C and 17α-hydroxyprogesterone, Fig. 5B, are, respectively, the only compounds in which the 20-keto group is either conjugated or very near a hydroxyl group and interactions between these groups might be expected to affect the shift of the adjacent methyl group. The other fluctuations of $\pm$1 c.p.s. easily could be due to variations in concentration. Although the shift of this group lies within two c.p.s. of the characteristic methyl shift of a 21-acetoxy-20-ketosteroid, careful measurements would appear to allow a distinction to be made between these two types of compounds.

21-Acetoxy-20-one Compounds.—As mentioned above, a near conflict exists between the methyl shift of the acetoxy group of these steroids and of the acetyl group of the 20-keto, C_{21}-steroids. Of five 21-acetoxy-20-one compounds studied, four were observed to give an acetoxy–methyl shift of 169 c.p.s. and the fifth gave a value of 168. No actual identity in the methyl shift has yet been observed for two compounds differing in the indicated way. If such were suspected, the resonance of the C_{21}-methylene group in the 21-acetoxy compounds could be used to settle the question. This resonance has such a distinctive shift value and structure that these combined features probably uniquely identify this group. What is observed

(20) Approximately 0.8 *M* solution in $CDCl_3$.

(21) H. S. Gutowsky and C. H. Holm, *J. Chem. Phys.*, **25**, 1228 (1956).

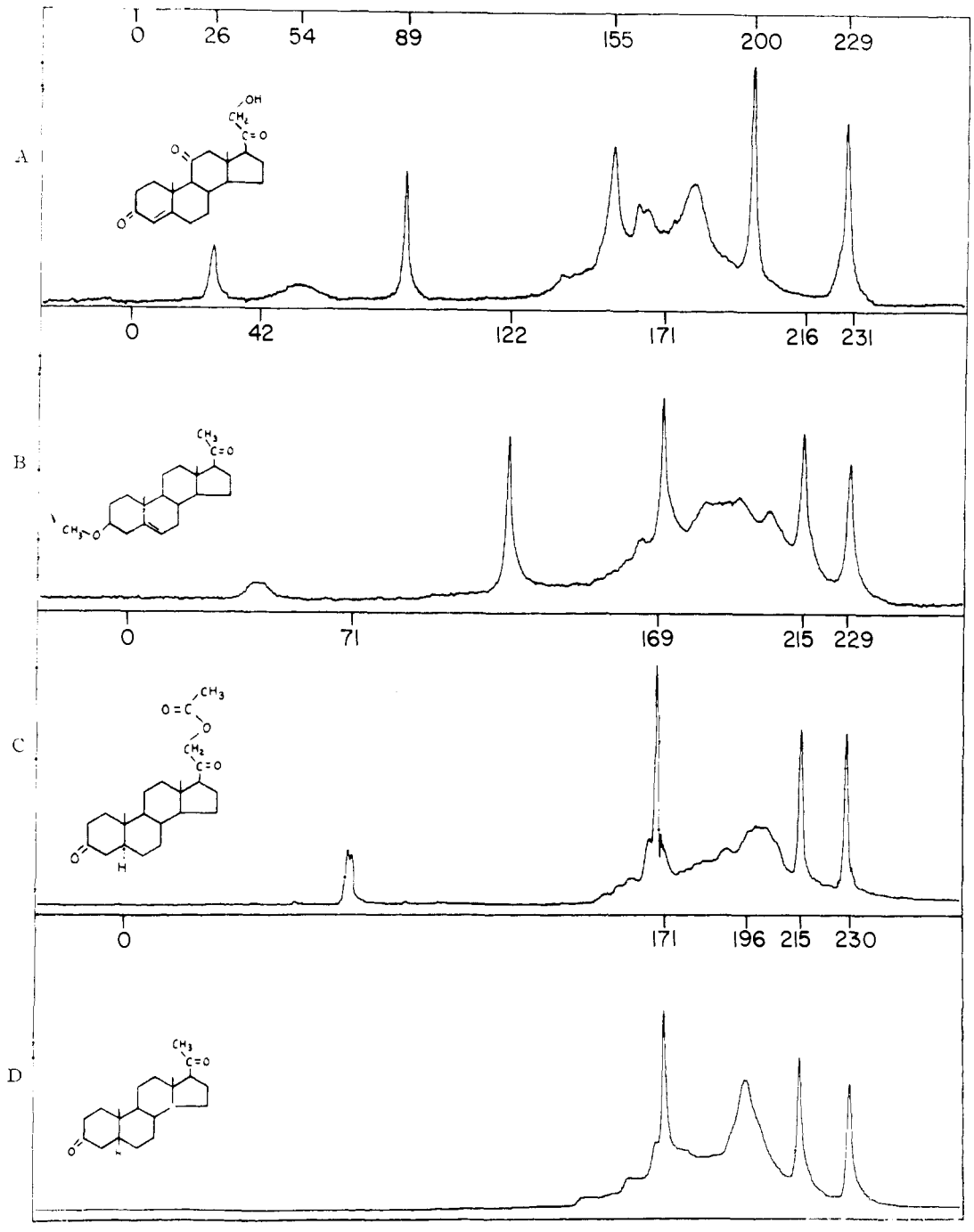

Fig. 6.—Nuclear magnetic resonance spectra in $CDCl_3$ solution: A, 11-dehydrocorticosterone (Δ^4-pregnene-21-ol-3,11,20-trione); B, pregnenolone-3-methyl ether; C, allopregnane-3,20-dione-21-acetate; D, pregnane-3,20-dione.

for the C_{21}-methylene resonance in the 5 compounds studied so far is a symmetrical quartet of lines with the inner pair separated by 1.5 to 2.0 c.p.s., and the outer pair separated by about 35 c.p.s. at 9395 gauss. The intensity of the inner pair is about 20 times that of the outer pair. The spacings of the inner pair and outer pair were found to be field dependent and each increased or decreased the same amount when the field was increased or decreased. The adjacent high and low intensity lines have a field-independent separation. All of this is consistent with the assignment of the lines to a pair of protons with a small chemical shift (δ about 0.175) and a large spin–spin coupling (J about 15.5 c.p.s.). The shift of the center of the pattern relative to the benzene reference is 70 ± 1 c.p.s. for all compounds studied. A typical example can be found in Fig. 6C.

The low shielding observed is consistent with the assignment of the lines to the C_{21}-methylene group since it is attached to both an oxygen and a carbonyl group. It is not unusual for the shifts due to two substituent groups to be roughly additive when the groups are both attached to a single meth-

ylene carbon atom. In this case, if an aliphatic hydrocarbon environment is taken as 220 c.p.s., the shift of methoxy to 122 c.p.s. (Fig. 6B) suggests that the effect of oxygen is a 98 c.p.s. shift to lower fields, while the shift of methyl ketones to 171 indicates that the carbonyl group shifts the methyl 49 c.p.s. in the same direction. Both groups acting together would give a resonance at 73 c.p.s. if strict additivity were expected. Furthermore, the high spin–spin coupling[22] also suggests that the two protons are attached to the same carbon atom, which again points to the CH_2 group. It is concluded that the situation in these molecules is something like that recently described by Nair and Roberts[23] in which the idea of unequal residence times in the various rotational conformations was shown to lead to a chemical shift between protons on the same carbon atom which was *not* averaged to zero, even when the potential energy barrier was not high enough to prevent rapid internal rotation of the groups relative to one another. It is interesting to note that a 21-hydroxy-steroid (Fig. 6A) did not show this behavior, its CH_2 resonance being a single sharp line at 89 c.p.s. The additional size of a 21-acetoxy group apparently leads to some steric interference which makes the residence times in the various rotational conformations unequal.

3β- and 11α-Acetoxysteroids.—The methyl resonances for the acetoxy group of eight compounds were measured and found to fall in the region 175 ± 2 c.p.s., half of this number being found exactly at 175 c.p.s. It would appear that these groups could be identified readily as a class and neither would interfere with observation of the characteristic resonances from 20-ketosteroids or 21-acetoxysteroids.

Angular Methyl Groups.—Accurate measurements of the shifts of the angular methyl groups appear capable of yielding considerable information concerning the detailed structure of the steroid. Each methyl group appears to be influenced mainly by structural variations in the nearby parts of the molecule, as might be expected. A full explanation of the reasons for the observed shifts might well involve a discussion of inductive shifts of electrons leading to changes in diamagnetic shielding, changes in second-order paramagnetic shielding due to local electron hybridization changes, long range shifts due to magnetic anisotropy associated with remote groups of electrons in the molecule, and very probably steric effects. Many of these terms are of the same order of magnitude but different sign and can be calculated only roughly. The observed shifts, as pointed out earlier, vary by only about 25 c.p.s. for the C_{18} protons and 28 c.p.s. for the C_{19} protons. Therefore, it seems unprofitable to attempt a detailed discussion of the shifts, but, instead, it may be worthwhile to make an empirical correlation of the observed shifts with known structural features.

Some of the angular methyl proton shifts observed when groups are introduced which are several bonds removed from C_{18} or C_{19} are large enough to suggest that the third mechanism considered above, namely, the fields arising at the angular methyl protons due to magnetic anisotropy of remote groups of electrons (not involved in bonding the angular methyl protons), is important. If this effect predominates, the shifts of several perturbing groups would be additive, since the fields due to them would simply superimpose at the angular methyl protons. Additivity is observed for most of the angular methyl shifts observed in this study. It would not be expected, *a priori*, for the other mechanisms considered above, since several groups acting *via* these various mechanisms upon the probability distribution and angular momenta of the bonding electrons of the angular methyl protons would not be likely to perturb the wave functions in a way which would lead to simple additivity.

(a) **C_{19}-proton Peaks at 219–221 c.p.s.**—A shift value of 220 ± 1 c.p.s. appears to be a maximum value for C_{19} protons achieved in steroids without carbon–carbon double bonds, keto-groups or other nearby substituents in the A, B and C rings. Substitution of hydroxyl at the C_3-position appears to have little effect, since the C_{19} proton shift differs by less than one c.p.s. from the value 220 c.p.s. for androsterone and epiandrosterone where the hydroxyl is α and β, respectively. Even a change from the *trans* A/B ring fusion (androsterone) to the *cis*-A/B fusion (5-isoandrosterone) produces only a 2 c.p.s. C_{19} proton shift.

An exception is 5,6-dihydroergosterol acetate with a C_{19}-proton shift assigned at 223 c.p.s. and a Δ^7-type structure. A possible explanation is that magnetic anisotropy of the double bond produces a significant shift, the direction being dependent on the relative orientation of the C_{19} methyl group and the double bond, and in this case resulting in increased shielding. A shift of similar magnitude (4 c.p.s.) is found upon the introduction of the Δ^7-group into cholesterol, relative to the shift in cholesterol itself.

(b) **C_{19}-Proton Peaks at 214–216 c.p.s.**—The C_{19}-proton shift of 220 ± 1 c.p.s. decreases 5 to 7 c.p.s. upon the introduction of a 3-keto group. The type of fusion of the A/B rings does not seem to affect the shift, the spectra of pregnane-3,20-dione and allopregnane-3,20-dione, shown in Figs. 6D and 7A, both giving C_{19} proton peaks at 215 c.p.s. A similar shift was observed in 11 different Δ^5-steroids which yield a C_{19}-proton shift of 215 ± 1 c.p.s. The actual distribution was 214 (3), 215 (6) and 216 (2).

In the event that the identification of an unknown steroid was attempted, based on these data, the 3-ketosteroids could be distinguished readily from the Δ^5-steroids by the presence in the spectrum of the latter of a peak at 42 ± 2 c.p.s. (see Table I).

If additivity of the shifts holds, the C_{19} proton peak in Δ^5-cholestene-3-one would be predicted to fall near 208 c.p.s. The peak is observed at 209 c.p.s. in this compound.

Three other groups have been observed to decrease the C_{19} proton resonance shift by 4–7 c.p.s., but only in compounds in which more than one perturbing group was simultaneously present. Again, if additivity is assumed to hold, from measurements

(22) The coupling of 15.5 c.p.s. is more than twice that of the CH^2 and CH_2 groups in ethyl alcohol, for example.

(23) P. M. Nair and J. D. Roberts, THIS JOURNAL, **79**, 4565 (1957).

of 3,11,20-triones it is found that the 11-keto group decreases the C_{19}-proton shift by approximately 7 c.p.s., and in a steroid with only the 11-keto group one would expect the C_{19} protons around 214 c.p.s. Similarly, from the C_{19}-proton shift of 205 c.p.s. in 17α,21-dihydroxy-Δ^1-allopregnene-3,11,20-trione-21-acetate, the effect of the Δ^1-group is suggested to be a decrease of about 3–4 c.p.s. in the C_{19} proton shift. Finally, the C_{19} proton shift at 202 c.p.s. in $\Delta^{9(11)}$-17-hydroxydesoxycorticosterone suggests that the $\Delta^{9(11)}$-group exerts an effect to decrease the shift by about 6 c.p.s. If only this group were present, the resonance might very well fall in the 214–216 c.p.s. range. Further work is, of course, indicated to determine the effects of these three groups in suitable compounds where additivity will not have to be assumed, and to determine whether other possible groups might lead to a C_{19} proton peak at 215 ± 1 c.p.s.

In identification work, the Δ^1-steroid could be double-checked by its distinctive C_1-proton shift (see Table II). The Δ^5- and $\Delta^{9(11)}$-steroids would present a difficulty, since the difference in the shifts for the protons attached to the doubly bonded carbons (see Table II) is hardly large enough to be considered conclusive. Finally, a criterion for assigning a 3-keto or 11-keto structure to a steroid with a C_{19}-proton peak near 215 c.p.s. may be the presence of a moderately strong, sharp peak, in the region 150–160 c.p.s., observed in five different 11-ketosteroids. This resonance is assigned to the C_{12} protons, unsplit by spin coupling owing to the lack of protons on adjacent carbon atoms.

(c) **C_{19}-Proton Peaks at 210–211 c.p.s.**—Only two examples of C_{19} shifts in this region were noted and these were 11 α-hydroxypregnane-3,20-dione, and the corresponding allo-compound. The 3-one-type compounds are known to give a peak at 215, hence the 11α-hydroxyl group is suspected of influencing the C_{19}-proton resonance position to the extent of only 4 or 5 c.p.s. No effect is noted on the C_{18} proton peak.

(d) **C_{19}-Proton Peak at 207–209 c.p.s.**—When both the 3-keto and 11-keto groups are present, or when the 3-keto group and double bond to C_5 are present, as in Δ^4-3-ones or Δ^5-3-ones, the effects are apparently additive and the C_{19}-proton resonance is found at 207–209 c.p.s. This leads to the prediction that Δ^4-11-ones and $\Delta^{5(6)}$-11-ones would show a similar shift. This prediction could not be verified for lack of suitable compounds during the present work. Further studies are indicated.

It is quite easy to determine which of the above steroid types is represented by the high resolution n.m.r. spectrum since the Δ^4-3-ones will always show a peak in the region 23–30 c.p.s., (see Table I) while the 3,11-diketosteroids will not, and the unconjugated Δ^4- or Δ^5-steroids should give a peak near 40 c.p.s.

The C_{18}-proton peak remains unaffected (within experimental error) in both Δ^4-3-ones and 3,11-diones.

(e) **C_{19}-Proton Peak at 204 c.p.s.**—The spectrum of 11 α-hydroxyprogesterone yields a C_{19}-proton peak at 204 c.p.s. Since this lies 4 c.p.s. lower than the expected value of 208 c.p.s. for a Δ^4-3-one, the effect of the 11α-hydroxyl group is again shown to be approximately 4 c.p.s., in agreement with the conclusion reached in part (c) of this section.

(f) **C_{19}-Proton Peaks at 199–200 c.p.s.**—Adrenosterone, 11-ketoprogesterone and 11-dehydrocorticosterone (Figs. 7B, 3C and 6A) give C_{19}-proton peaks at 199, 200 and 200 c.p.s., respectively. This provides another example of the apparent additivity of the effects of 3- and 11-keto groups, and double bonds to C_5. When all three are present, the C_{19}-proton resonance lies 19–21 c.p.s. closer to the reference than in steroids in which these groups are absent.

(g) **C_{19}-Proton Peaks at 197 c.p.s.**—Corticosterone, Fig. 7C, 11β-hydroxyprogesterone, Fig. 7D, and 11β-21-dihydroxy-4,17(20)-pregnadiene-3-one all yield spectra with C_{19}-proton peaks at 197 c.p.s. The effect of an 11β-hydroxyl group is therefore suggested to be about 2–3 c.p.s. larger than that of an 11-keto group, which had previously been deduced to be 7 c.p.s. The 11β-hydroxyl group is, therefore, provisionally assigned an ability to shift the C_{19} protons by 10 c.p.s. toward the reference. It is interesting to note that in corticosterone and 11β-hydroxyprogesterone the C_{18}-proton peak also lies 10–11 c.p.s. below the corresponding peak in the 11-keto compounds. Molecular models show that the 11β-hydroxyl group lies approximately between the angular methyl groups and should interact strongly with both, while in the 11-keto or 11α-hydroxyl cases the perturbing group lies much closer to C_{19} than to C_{18}. This is in qualitative accord with the observed n.m.r. shifts.

(h) **C_{19}-Proton Peak at 193 c.p.s.**—The C_{19}-proton peak position observed in 9α-fluorocorticosterone acetate (Fig. 5C) can be explained tentatively by attributing to the 9α-fluorine atom the ability to shift the C_{19}-proton resonance an additional 4 c.p.s. It would be interesting to examine other 9α-fluorosteroids to determine whether the additivity observed for other groups includes the 9α-fluorine atom. The C_{18}-proton peak is found at the same position as in corticosterone.

(i) **C_{18}-Proton Peaks at 228–234 c.p.s.**—Many of the steroids studied in the present work are 20-keto-C_{21}-steroids. The C_{18}-proton peak of these compounds falls in the range 228–234 c.p.s. unless there is an 11β-hydroxyl group present or a double bond (olefinic or ketonic) to C_{17}. These two exceptions will be considered in following parts of this section. Compounds without the 20-keto group, such as cholesteryl acetate, stigmasteryl acetate and 5,6-dihydroergosteryl acetate also yield C_{18}-proton resonances in this region. This would seem to indicate that the 20-keto group is not effective in shifting the C_{18}-proton peak. In spite of this lack of effect on the C_{18} peak, the 20-keto group can be identified in steroids by the characteristic C_{21}-methyl peak (see section on 20-keto-C_{21}-steroids) at 171 ± 1 c.p.s., by the methylene peak at 89 c.p.s. in 21-hydroxy-20-ketosteroids, or by the methylene multiplet (see section on 21-acetoxy-20-one steroids) at 70 ± 1 c.p.s.

(j) **C_{18}-Proton Peaks at 223–224 c.p.s.**—Testosterone and 19-nortestosterone yield C_{18}-proton peaks at 224 and 223 c.p.s., respectively. The 17β-

hydroxyl group is therefore assigned an ability to shift the C_{18}-proton peak about 8 c.p.s. by comparison with progesterone. Unfortunately, other 17β-hydroxysteroids were not on hand, so that no additivity relationships involving this group could be tested. However, 4-chloro-17α-hydroxypregnane-3,11,20-trione was studied and the C_{18}-proton peak was found at 234 c.p.s. It appears, therefore, that the β-orientation of the 17-hydroxyl group is more effective in lowering shift value of the C_{18}-protons than the α-orientation by about 10 c.p.s.

Since the 11β-hydroxyl group also lowers the shift value of the C_{18}-protons by 8–10 c.p.s., it is possible that a C_{18}-proton peak at 223–224 c.p.s. may not always denote a 17β-hydroxyl group. However, there are, as usual, cross checks which can be applied. First, there is quite a difference in the hydroxyl resonance of 11-hydroxy and 17-hydroxysteroids (see section on hydroxyl peaks). Second, the 17β-hydroxy compounds give a fairly well-defined triplet at 109–110 c.p.s. (Fig. 2A) from the proton on C_{17} which is apparently spin–spin coupled to the C_{16}-methylene protons. Finally, if anything is known about the substituents on the A or B rings, it will often be possible to decide from the C_{19}-proton peak position whether or not an 11β-hydroxyl group could be present.

(k) **C_{18}-Proton Peaks at 220–222 c.p.s.**—Six 17-ketosteroids were studied and these C_{18}-proton positions found: 223(2), 222(3), and 220(1). The 17-keto group therefore appears to lower the C_{18}-proton shift by 10–12 c.p.s. The same shift was found for one other compound, namely, 16-dehydropregnenolone, whose C_{18}-proton peak appears at 220 c.p.s. It may be possible to expect this 10–12 c.p.s. effect for all steroids in which C_{17} is forming a double bond.

The 11β-hydroxyl group in 11β-hydroxyprogesterone (Fig. 7D) results in a C_{18}-proton peak at 220 c.p.s. which could be confused with a 17-keto structure. However, the C_{19} peak at 197 c.p.s. strongly suggests an 11β-hydroxyl group; furthermore, the methyl peak at 171 is quite characteristic of the C_{21}-methyl in a 20-ketosteroid.

The C_{18}-proton peak in 17α-methyltestosterone (Fig. 1B) also appears at 220 c.p.s. Although in an unknown compound it would be tempting to assert the presence of a 17-keto group on the basis of this information, the temperature-dependent peak at 176, indicating the presence of a hydroxyl group, and the high amplitude of the peak at 208 c.p.s., indicating an extra methyl group in the steroid, might call attention to the need for caution, since they might very well be located on C_{17}, as is actually the case.

(l) **C_{18}-Proton Peaks below 220 c.p.s.**—Corticosterone and 9α-fluorocorticosterone give C_{18}-proton peaks at 218 c.p.s. When the 11β-hydroxy group is removed as in desoxycorticosterone acetate the C_{18}-proton peak is found at 228 c.p.s. The 10-11 c.p.s. lowering of the C_{18}-proton shift value by 11β-hydroxyl groups proposed in part (g) of this section is thus given added weight.

It was considered worthwhile to attempt to predict the shift values for the previously unmeasured C_{18}- and C_{19}-proton peaks in the compound 11β-21-dihydroxy-4,17(20)-pregnadiene-3-one. The corresponding compound without the 11β-hydroxyl group or C_{17}-double bond would give a C_{18}-proton peak at 228–230 c.p.s. and a C_{19}-proton peak at 207–208 c.p.s. The 11β-hydroxyl would be predicted to move the C_{19}-proton peak by about 10 to 197–198 c.p.s. The observed value was 197 c.p.s. A similar movement of the C_{18}-proton peak would be expected, but if additivity of the effects of two perturbing groups is expected, the $\Delta^{17(20)}$-group would also contribute about 11 c.p.s. and it was therefore predicted that the C_{18}-proton peak would fall at 208 c.p.s. The observed value was 209 c.p.s. This is 9 c.p.s. lower than any other C_{18}-proton peak encountered in this study.

Chemical Shifts Due to Conformation.—Substituent groups in steroids may be oriented either α or β to the ring system. In the cholestane series a 3β-hydroxyl group is equatorial, while in the coprostane series it is axial. If an unknown steroid were known to fall into one or the other of these two series, it would be possible to determine the orientation of the substituent if the conformation of either the group or the proton on the same carbon atom could be related to their respective n.m.r. shifts. Some of the resonance peak positions were examined with this end in view.

Some work has been reported[24] by Lemieux, Kullnig, Bernstein and Schneider with acetylated carbohydrates in which shifts of 5–10 c.p.s. between the signals for methyl hydrogens of equatorial and axial acetoxy groups were assigned. A shift of approximately 8 c.p.s. was assigned to the difference between axial and equatorial hydrogens.

Axial and equatorial protons in steroids cannot be observed unless they can be distinguished from the remaining skeletal protons. Fortunately, the attachment of oxygen directly to the same carbon atom results in a large shift of the proton resonance toward lower fields. The resonance can then be observed without interference. Spin–spin coupling to protons on neighboring carbon atoms may split the resonance into a number of components which, because of the typical broadening associated with these skeletal protons, usually results in a low, broad hump. Unless a fairly concentrated solution can be studied, the noise level may obscure the signal and prevent an accurate shift measurement.

The shift value for the proton on the hydroxyl-substituted carbon atom was measured in a number of hydroxylated steroids. These values are reported in Table III. For protons attached to C_3, the axial shift was 22–25 c.p.s. higher than the equatorial. When the configuration is inverted at C_5 in going from androsterone to 5-isoandrosterone, the proton on C_3 is converted from equatorial to axial. The shift then becomes the same as for the axial proton in epiandrosterone, which suggests that it is the *local* environment which is most important, and not the relationship of the proton to the entire steroid framework.

The proton adjacent to an acetate group usually lies about 45 c.p.s. lower than in the case of a corresponding hydroxyl group. Acetates with both axial and equatorial conformations at the same car-

(24) R. U. Lemieux, R. K. Kullnig, H. J. Bernstein and W. G. Schneider, THIS JOURNAL, **79**, 1005 (1957).

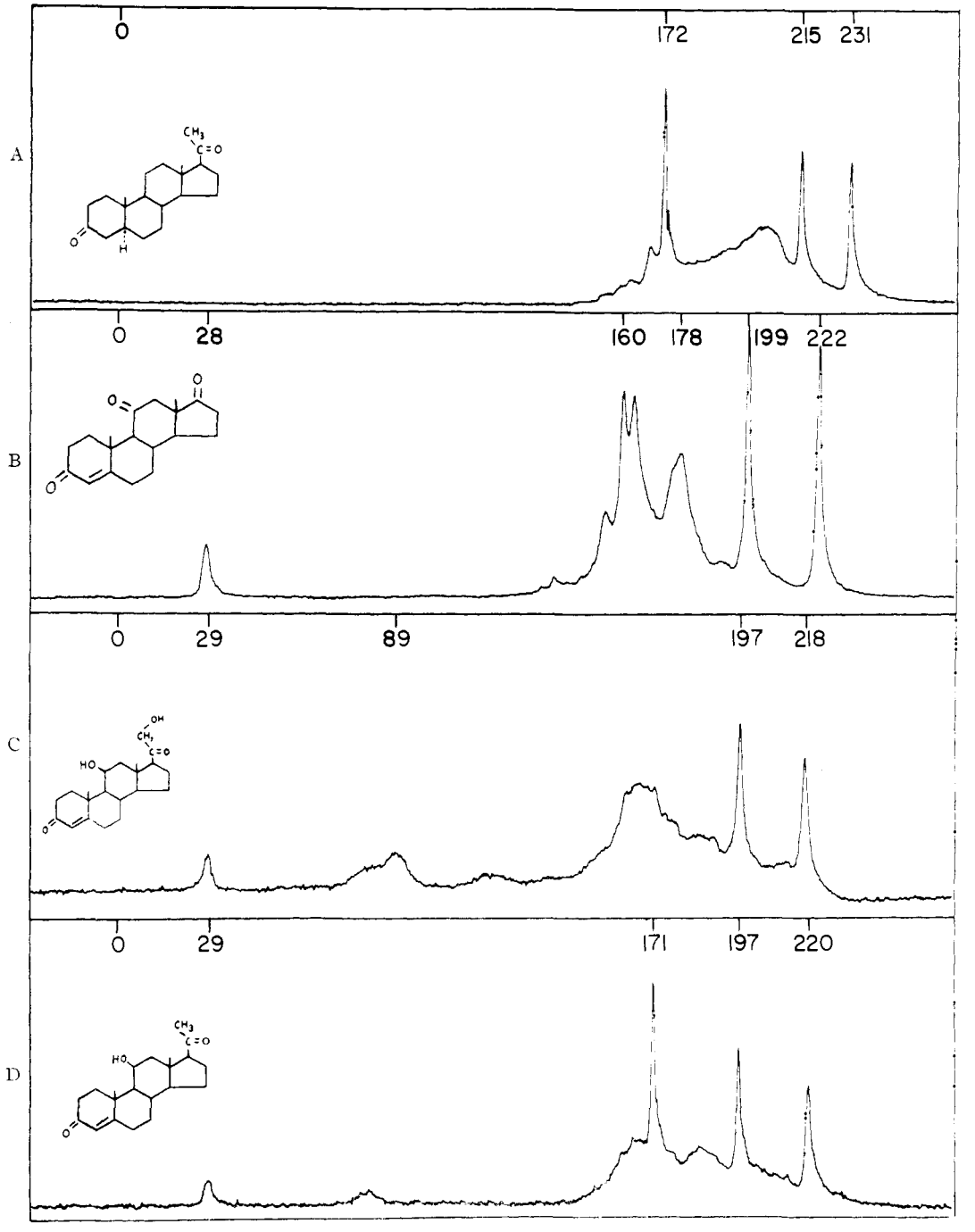

Fig. 7.—Nuclear magnetic resonance spectra in $CDCl_3$ solution: A, allopregnane-3,20-dione; B, adrenosterone (Δ^4-androstene-3,11,17-trione); C, corticosterone (Δ^4-pregnene-11β,21-diol-3,20-dione); D, 11β-hydroxyprogesterone.

bon atom should show a similar effect. An axial acetoxy steroid, 2β-acetoxycholestane,[25] showed no shift in the acetoxy methyl resonance relative to the value of 175 ± 2 c.p.s. found for eight equatorial (3β- and 11α-) acetoxy steroids, although such a shift had been hoped for in view of the work of Lemieux, *et al.*[24]

Conclusion

The unique ability of proton magnetic resonance studies to reveal in molecules structural details which can be related unequivocally to the presence or absence of specific chemical shift values should be very useful, particularly if those structural details do not result in interpretable perturbations of the characteristic group frequencies in the infrared, visible or ultraviolet spectra. Proton–proton spin coupling, giving rise to readily interpreted multiplet structures in the n.m.r. spectra, should further enhance such usefulness, especially for establishing the numbers of protons on adjacent carbon atoms. As a means of determining the number of methyl groups and to some extent their position on the

(25) This sample was provided through the kind coöperation of Prof. W. G. Dauben of the University of California.

TABLE III

N.M.R. SHIFTS FOR PROTON ON SUBSTITUTED CARBON ATOM IN STEROIDS

Compound	Proton location	Substituent and orientation	Proton conformation	Shift, c.p.s.
Androsterone	C_3	3α-OH	Equatorial	91
Epiandrosterone	C_3	3β-OH	Axial	113
5-Isoandrosterone	C_3	3α-OH	Axial	113
3α-Hydroxypregnane-11,20-dione	C_3	3α-OH	Axial	116
11β-Hydroxyprogesterone	C_{11}	11β-OH	Equatorial	78
9α-Fluorocorticosterone acetate	C_{11}	11β-OH	Equatorial	77
Corticosterone	C_{11}	11β-OH	Equatorial	82
11β-21-Dihydroxy-4,17-(20)-pregnadiene-3-one	C_{11}	11β-OH	Equatorial	78
11α-Hydroxyprogesterone	C_{11}	11α-OH	Axial	95
11α-Hydroxypregnane-3,20-dione	C_{11}	11α-OH	Axial	95
11α-Hydroxyallopregnane-3,20-dione	C_{11}	11α-OH	Axial	100
19-Nortestosterone	C_{17}	17β-OH	(Axial)	110

steroid framework, the n.m.r. method may very well be unrivaled. Although based on empirical correlation with laboriously established known structures, the characteristic chemical shift values associated with axial and equatorial protons should be helpful in cases where a rapid determination of the orientation of a substituent is desired.

The ability to work with a few milligrams of material will allow practical application as well as purely academic studies, although it should be noted that when the sensitivity is pushed to the limit, the esthetic appearance of the spectra will deteriorate, and information associated with the weaker lines will be lost. Samples between 10 and 50 mg. should permit spectra similar to those displayed in the accompanying figures to be obtained. Under these conditions, application of nuclear magnetic resonance techniques to identification problems and structural determinations in steroid chemistry should prove of considerable value in conjunction with the established chemical and physical methods.

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Steroid–Protein Interactions. V. Comparison of Spectrophotometric and Equilibrium-dialysis Procedures for Determination of Binding Constants[1]

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The validity of a spectrophotometric procedure for the determination of interaction between proteins and Δ^4-3-ketosteroids was examined by a comparison with the established method of equilibrium dialysis. Values of $r/[S]$, *i.e.*, moles of Δ^4-3-ketosteroid bound per mole human serum albumin, per mole of free steroid, have been calculated from spectrophotometric data for testosterone, progesterone, desoxycorticosterone and cortisol. These binding values were found to be in agreement with those determined by equilibrium dialysis.

Introduction

It has long been known that the absorption spectra of certain molecules are altered in the presence of proteins.[2] This spectral change is indicative of an interaction between the two components; it usually consists of a decrease of the extinction coefficient and may also be connected with a hypsochromic or bathochromic shift of the absorption maximum. This phenomenon has been observed and utilized particularly in the visible range of the spectrum; it constitutes the basis of spectrophotometric methods to measure intermolecular interactions, *e.g.*, with dyestuffs.[2] Analogous findings also have been reported for the ultraviolet range.[3–5]

Recent observations have shown that the ultraviolet absorption spectra of Δ^4-3-ketosteroids are altered in the presence of various proteins.[6] In some cases, the absorption maximum is shifted toward shorter wave lengths; in all cases where interaction takes place, the extinction coefficient ϵ is decreased. The extent of the reduction of ϵ has been found to be approximately proportional to the strength of interaction determined by various other methods.

The spectrophotometric procedure for the determination of interaction between Δ^4-3-ketosteroids and proteins[6] is a convenient technique which permits a rapid analysis of binding behavior in a system free of interference by components other than the solvent and the two interacting solutes. However, in contrast to other methods, *e.g.*, equilibrium dialysis, electrophoresis, solvent distribution, ultracentrifugation and others, the spectrophotometric method does not give information on binding by a physical separation of the free and bound portion of the molecules under investigation. It appeared necessary, therefore, to test the validity of the use of spectral data as an indicator of strength of interaction. In the present study, values for binding

(1) For paper IV of this series see ref. 8.

(2) I. M. Klotz, Chapter 8, "Protein Interactions." In "The Proteins," H. Neurath and K. Bailey, Eds., Vol. I, part B, Academic Press, Inc., New York, N. Y., 1953, p. 727.

(3) H. Theorell and R. Bonnichsen, *Acta Chem. Scand.*, **5**, 1105 (1951).

(4) N. B. Madsen and C. F. Cori, *J. Biol. Chem.*, **224**, 899 (1957).

(5) R. C. Warner, *ibid.*, **229**, 711 (1957).

(6) U. Westphal, *Arch. Biochem. & Biophys.*, **66**, 71 (1957).