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STRUCTURE AND MECHANISM OF FORMATION OF THE TWO FORMS OF 18-HYDROXY-11-DEOXYCORTICOSTERONE

ASIT K. ROY, LEYLA C. RAMIREZ and STANLEY ULICK*

Veterans Administration Hospital, Bronx, New York, 10468, and the Department of Medicine, Mount Sinai School of Medicine of the City University of New York, U.S.A.

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SUMMARY

18-Hydroxy-11-deoxycorticosterone (18-OH-DOC) has been observed to exist in two interconvertible forms of markedly different chromatographic mobility. The structure of these forms has now been established. The more polar form is the cyclic hemiketal and the less polar is a mixed ketal at C-20 derived by reaction with an alcoholic solvent. This unusually facile ketalization is catalyzed by traces of acidic impurities present in most commercial sources of reagent grade methanol or ethanol, but can be abolished by removal or neutralization of these impurities.

INTRODUCTION

18-Hydroxy-11-deoxycorticosterone (18-OH-DOC) was first identified in adrenal incubates by Kahnt *et al.* [1], synthesized by Pappo[2], and identified as a major corticosteroid in the rat adrenal by Birmingham and Ward[3] and by Péron[4]. In that species, 18-OH-DOC arises from the fasciculata zone of the adrenal cortex [5, 6] and is ACTH-dependent [7]. In man, where 18-OH-DOC is much less abundant, the normal rate of secretion of approximately 100 μg/day is also stimulated by ACTH [8, 9] but not by the renin-angiotensin system [9]. Despite its chemical similarity to 18-hydroxycorticosterone, 18-OH-DOC is not an important precursor of aldosterone [10] since it arises in a zone which lacks the terminal oxidase mechanism for aldosterone synthesis. Its activity as a mineralocorticoid is quite low [11–13]. In searching for a role for 18-OH-DOC, some investigators have implicated the steroid in genetic and experimental hypertension in rats [8, 14–16], and Melby and coworkers [8] have reported that some patients with benign essential hypertension with suppressed plasma renin activity have increased excretion of the tetrahydro metabolite of 18-OH-DOC. Messerli and coworkers [17] reported elevated secretory rates of 18-OH-DOC in benign essential hypertension generally, including those with normal as well as low plasma renin activity. We did not find increased 18-OH-DOC secretion in a small series of low renin hypertensives [9].

A disconcerting property of 18-OH-DOC, complicating its estimation in body fluids and glandular extracts, is its tendency, first described by Dominguez[18], to exist in two apparently interconvertible forms of markedly different chromatographic mobility. Of the several mechanisms suggested for this phenomenon, tautomeric equilibrium between α-ketol and cyclic hemiketal (I) forms appears to have been more generally accepted. The instability and interconvertibility of these forms has made it difficult to determine their exact structure, and at recent discussions [16, 19] the problem was not resolved.

A renewed approach to the problem became possible when we observed that acetylation stabilized the forms of 18-OH-DOC and thus allowed chromatographic purification on paper and on alumina columns, and subsequent assignment of structure by infrared and mass spectroscopy. By this means we have demonstrated that the less polar form was not a tautomer of 18-OH-DOC but actually a C-20 ketal derivative formed by reaction of the cyclic hemiketal form of the steroid with an alcoholic solvent and catalyzed by traces of acidic impurities. The tendency for these ketal derivatives to hydrolyze spontaneously to the hemiketal form when rechromatographed led previous investigators to conclude that tautomeric interconversion was taking place.

METHODS AND MATERIALS

Solvents. Absolute methanol (Fisher Scientific, analytical reagent) was redistilled. Ketal formation was also observed with methanol from other manufacturers. Ethanol (95%) from U.S. Industrial Corp. was redistilled. Ketal formation with either alcohol was abolished by distillation from sodium hydroxide pellets or by the addition of an amine.

Steroids. 18-OH-DOC [2] was a gift of Dr. R. Pappo.

Tritium-labeled 18-OH-DOC was prepared by catalytic exchange. In initial experiments, the 21-acetate derivative was found to give better yields of tritiated product than the free steroid. We subsequently learned that the free steroid could be labeled directly

*To whom correspondence should be addressed.

if a major tritiation product, the C-20 ketal derivative, was hydrolyzed with 0·1N HCl, as described below.

In a typical preparation, 1·0–5·0 mg of steroid in dimethylformamide was exchanged with 10 Ci tritiated water in the presence of a rhodium on alumina catalyst at 50° for 16 h (performed by New England Nuclear Corp.). Methanol-exchangeable hydrogen isotope was removed and the product chromatographed preparatively on the dichloroethane:toluene (1 : 1 v/v) system once with formamide as stationary phase and twice with ethylene glycol as stationary phase, after which it was homogeneous by radioactive scanning. When carrier steroid was added, the U.V.-absorbing and radioactive steroid migrated together as free 21-monoacetate and etiolactone derivatives. The final specific activity, determined from the $^{3}H/^{14}C$ ratio of the monoacetate derivative prepared from ^{14}C-acetic anhydride was 0·9 mCi/μmol.

Chromatography. Paper systems used formamide or glycols as stationary phase, applied by dipping in a 25% solution in acetone. The preparation of neutral low activity alumina for columns was described previously [20] (Table 1).

Spectroscopy. For infrared spectroscopy, a solution containing approximately 25 μg steroid was evaporated in a 2 ml conical test tube. Spectroscopic grade KBr (5·0 mg) (Harshaw Chemical Co.) was added along with spectroscopic grade methylene chloride to disperse the steroid on the salt. The solvent was evaporated under a stream of nitrogen, the residue was dried in a vacuum drying apparatus at 80°, and the pellet pressed in a carpenter's vice using a Beckman 1 × 5 mm die. Spectra were recorded on a Beckman Acculab 4 infrared spectrophotometer. The slight elevation above baseline in the spectra of compounds VI and VII was also present in a blank pellet and was therefore not due to a hydroxyl group in the sample.

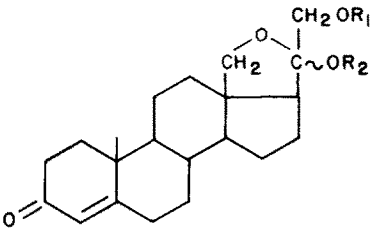
Mass spectroscopy was performed by Dr. Rodger L. Foltz of the Battelle Columbus Laboratories. Electron impact spectra were obtained on an A.E.I. MS 9 spectrometer and chemical ionization spectra on a Finnegan model 1015 using isobutane as reactant gas.

Table 1. Chromatographic properties of derivatives of 18-OH-DOC

Derivative	Chromatography		
	Migration	Reference	System
18-OH-DOC (I)	0.65	B	1
"	0.70	B	2
21-acetoxy (II)	0.6	DOC	3
20-methoxy (III)	0.8	DOC	3
20-ethoxy (IV)	1.1	DOC	3
20-methoxy-21-acetoxy (V)	1.0	Prog.	4
20-ethoxy-21-acetoxy (VI)	1.3	Prog.	4
20,21-diacetoxy (VII)	1.1	DOC	3

Systems: 1. Toluene : dichloroethane (1 : 1)/formamide; 2. Toluene : dichloroethane (1 : 1)/ethylene glycol; 3. Methylcyclohexane : toluene (1 : 1)/formamide; 4. Methylcyclohexane/formamide.

Reference steroids: B = corticosterone; DOC = 11-deoxycorticosterone; Prog = progesterone.



	R_1	R_2
I	H	H
II	CH_3CO	H
III	H	CH_3
IV	H	CH_2CH_3
V	CH_3CO	CH_3
VI	CH_3CO	CH_2CH_3
VII	CH_3CO	CH_3CO

Fig. 1.

RESULTS

Standard solutions of 18-OH-DOC that had been stored in distilled 95% ethanol in the refrigerator for several weeks or more showed, in addition to the polar form on chromatographic analysis, variable amounts of a less polar form, which on elution and rechromatography, reverted largely to the more polar form. When an ethanolic solution of 18-OH-DOC containing both forms was evaporated and the residue acetylated, the two principal products on chromatographic analysis were the 21-monoacetate of 18-OH-DOC (II) (Fig. 1) and larger amounts of a less polar monoacetate (VI) which, unlike the less polar form of 18-OH-DOC before acetylation, was now quite stable to repeated chromatography on paper and on alumina columns. This stability permitted the preparation of a chromatographically pure sample of the less polar monoacetate and spectroscopic assignment of structure as the 20-ethyl ketal 21-monoacetate derivative (VI). Thus the structure of the unstable less polar form before acetylation must have been the 20-ethoxy derivative (IV) formed by reaction with ethanol. Methanolic solutions of 18-OH-DOC were found to form the corresponding methyl ketals. The structure of these ketal and ketal acetate derivatives was verified by comparison with synthetic spectroscopically identified derivatives described under Syntheses.

Semiquantitative survey of reaction variables. The effect of reaction conditions on the reaction of 18-OH-DOC with alcohols was evaluated by paper and thin-layer chromatography of the U.V.-absorbing products and comparison with synthetic derivatives.

Anhydrous alcohols reacted faster than those containing water and methanol reacted faster than ethanol. Either distilled reagent grade methanol or redis-

Table 2. Effect of solvent purity on reactivity of 18-OH-DOC (10 μg/ml) with alcohols

Solvent	Conditions	Yield of C-20 ketal (%)
Methanol, distilled	Room temp 16 h	10
"	Reflux 1 h	50
+ 0.1% triethylamine	Reflux 1 h	0
distilled from NaOH	Reflux 1 h	0
+ acetic acid (0.1 M)	Room temp 4 h	100
Ethanol, 95% distilled	Reflux 1 h	30
+ 0.1% triethylamine	Reflux 1 h	0
Ethanol, 95% distilled from NaOH	Reflux 1 h	0
Absolute ethanol + acetic acid (0.1 M)	Room temp 4 h	90

tilled 95% ethanol reacted with 18-OH-DOC to form the corresponding ketal. Ketal formation was observed after storage of standard solutions in alcohols in the refrigerator for several weeks but was of course more rapid at room or reflux temperature. Dilute solutions of the steroid in alcohol (10 μg/ml or less) yielded greater percentages of the ketal derivative whereas more concentrated solutions (greater than 1 mg/ml) were more stable. The reaction was markedly accelerated by adding a trace of acid.

Effect of solvent purity on reactivity of 18-OH-DOC with alcohols. Table 2 shows that 18-OH-DOC reacts with reagent grade methanol to yield the C-20 ketal derivative. The reaction is accelerated by heat or the addition of a trace of acid but abolished by distillation of the alcohol from sodium hydroxide pellets or by the addition of an amine. Distilled, 95% ethanol reacted at reflux temperature with the steroid but ketal formation was similarly abolished when the alcohol was purified by distillation from sodium hydroxide pellets. These observations indicate that ketal formation with alcoholic solvents depends on the presence of acidic impurities since the reaction can be abolished by measures which remove or neutralize these impurities. The acid-catalyzed formation of ketals occurred with higher alcohols as well.

Selective removal of the C-20 ketal group. The C-20 ketal group of either the 21-hydroxy or 21-acetate derivatives could be selectively hydrolyzed under very mild acidic conditions that were similar to those used to remove the C-18-hemiacetal acetate of aldosterone or tetrahydroaldosterone [20]. A solution of the derivative containing amounts up to 1 mg was evaporated in a stoppered test tube, 10 ml 0·1N HCl added and the mixture was shaken mechanically for 30 min at room temperature. The hydrolyzed product was recovered by extraction with methylene chloride and the extract washed with 5% Na_2CO_3 and water, dried and evaporated. Chromatographic analysis indicated quantitative and selective regeneration of the C-20 hemiketal.

Reaction sequence. The sequence shown in Fig. 2 provided evidence for the structure of the reaction products of 18-OH-DOC with alcohols. In one sequence, the acid-catalyzed reaction with ethanol or methanol was carried out first, followed by acetylation, to give ketal acetate V or VI. Mild acid hydrolysis afforded the 21-monoacetate, II, of 18-OH-DOC. Alternatively, 18-OH-DOC was first acetylated to give monoacetate II, followed by acid-catalyzed reaction with ethanol or methanol to give the same ketal acetate obtained by the other sequence. Ketal formation, however, occurred more rapidly and in greater yield from the 21-alcohol than from the 21-acetate.

Synthesis of 18-OH-DOC derivatives

18-OH-DOC was mixed with the ^{3}H-labeled steroid to facilitate localization of reaction products on chromatograms by radioactive scanning.

Methyl or ethyl ketal derivatives. 18-OH-DOC (10 μg/ml) in methanolic or ethanolic acid (0·1M) reacts quantitatively at room temperature to form the corresponding methyl or ethyl ketal. The reaction is virtually complete after 4 h. For larger concentrations of steroid (2·0 mg/ml) the reaction mixture was heated under reflux for 1 h. Evaporation of the solvent and chromatography of an aliquot of the product on a silica gel plate developed with 3% methanol in dichloroethane revealed a single component representing the corresponding methyl or ethyl ketal. Isolation was usually not attempted unless the 21-acetate group was present because of decomposition to the hemiketal during elution and rechromatography.

18-OH-DOC-21-acetate. (21-acetoxy-20-hydroxy-18,20-epoxy-4-pregnen-3-one) (II). Crystalline 18-OH-DOC (0·1–1·0 mg) was treated with 0·2 ml dry pyridine and 0·05 ml acetic anhydride for 16 h at room temperature and excess reagents were removed under vacuum. Chromatographic analysis of an aliquot showed a single product (II) which migrated as one spot under paper system 2 (Table 1) and on a silica gel thin layer plate developed with 3% methanol

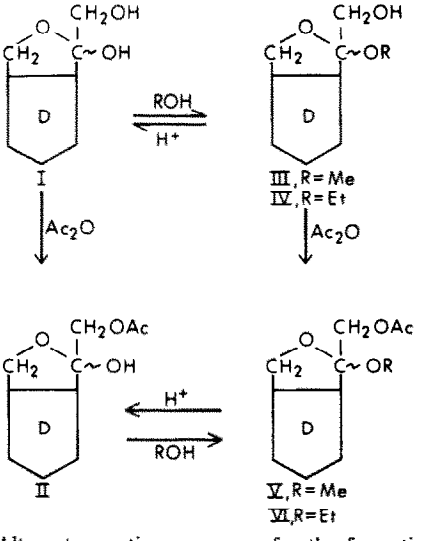


Fig. 2. Alternate reaction sequences for the formation of 18-OH-DOC derivatives.

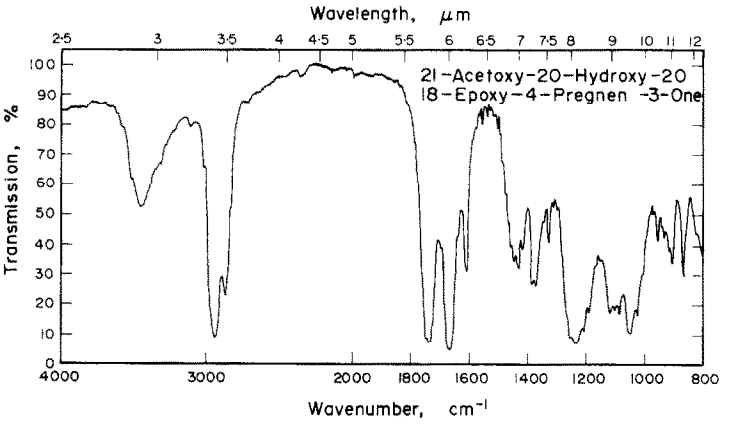


Fig. 3.

in ethylene dichloride (R_f = 0·25). One acetyl group per mol was indicated by the $^3H/^{14}C$ ratio of a sample prepared from ^3H-18-OH-DOC and ^{14}C-acetic anhydride of known specific activities (found 11·5; theoretical for a monoacetate, 11·2). Like 18-OH-DOC itself [1], the monoacetate (II) gave a positive Porter–Silber test. The position of the acetate at C-21 rather than C-18 was suggested by the slow rate of reduction of alkaline blue tetrazoleum, characteristic of the 20, 18-cyclic hemiketal form of 18-OH-corticosteroids [21] and was confirmed by spectroscopic analysis and by oxidation to 21-acetoxy-3,20-dike-to-4-pregnen-18-oic acid by chronic oxide-pyridine complex [22].

Spectroscopic data were obtained after a minimum amount of handling because of facile hydrolysis of the acetate to 18-OH-DOC. Infrared spectroscopy (Fig. 3) showed the presence of conjugated ketone (6·00 μm) and ester groups (5·75 μm) and the hydroxyl (2·90 μm) of the hemiketal form. There was no evidence of a 20-ketone or of the characteristic shift in ester absorption due to a 21-acetoxy-20-ketone structure [23]. Chemical ionization mass spectroscopy (Fig. 4) showed peaks due to the protonated molecular ion and fragments due to loss of water and of acetic acid. The electron impact mass spectrum showed similar fragments but no molecular ion.

Stability of 18-OH-DOC-21-monoacetate. When the monoacetate II was eluted from system 3 and rechromatographed, considerable hydrolysis to the free steroid was observed. Substitution of ethylene or propylene glycol for formamide as the stationary phase in system 3 resulted in somewhat greater stability. Spontaneous hydrolysis was also observed on thin layer silica gel chromatography (mobile phase: 3% methanol in dichloroethane) if the plate was not developed immediately after application of the sample. The monoacetate was relatively stable in solution at concentrations greater than 0·2 mg/ml when stored in the refrigerator for 2 weeks in 95% ethanol. Stability decreased with decreasing steroid concentration and increasing water content. At a concentration of 10 μl/ml in 70% ethanol there was 60% hydrolysis after 16 h at room temperature (Table 3).

18-OH-DOC acetate in reagent grade absolute methanol underwent slight hydrolysis at room or reflux temperature, but ketal formation occurred simultaneously so that only the ketal form but not the free steroid was detected. When acidic impurities were removed from the solvent, either by distillation from base or addition of an amine, the simultaneous hydrolysis of the 21-acetate and ketalization was actually accelerated (Table 3). Thus, the 21-acetate was actually more stable in the presence of traces of acidic

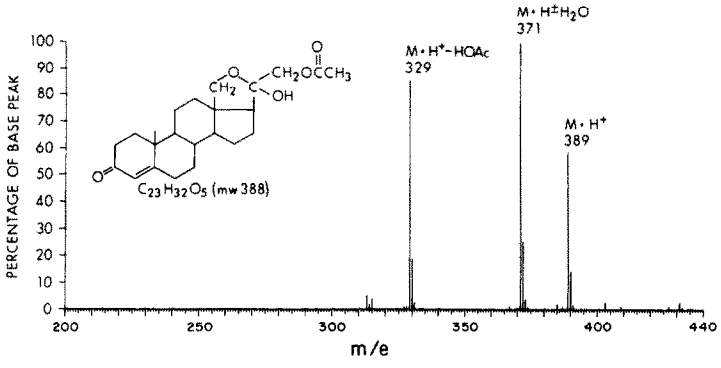


Fig. 4. Chemical ionization mass spectrum of 18-OH-DOC-21-monoacetate.

Table 3. Stability and reactivity of 18-OH-DOC-21-acetate (10 μg/ml)

Conditions		Products %		
Solvent	Temperature/ Time	Ketal	Unreacted	Other
Methanol	25°, 3 days	5	95	
"	Reflux, 1 h	10	90	
+ triethylamine (0.1%)	25°, 3 days	90		10 (18-OH-DOC)
distilled from NaOH	Reflux, 1 h	90		10 (18-OH-DOC)
Methanolic acetic acid (0.1 M)	25°, 1 h		50	50 (ketal acetate)
95% ethanol	10°, 2 weeks	trace	95	
70% ethanol	25°, 16 h		40	60 (18-OH-DOC)

impurities. The stability of the acetate toward acid was further demonstrated by the reaction with methanolic acetic acid. At reflux temperature for one hour, the 21-acetate was not removed but a 50% yield of the 20-methyl-ketal 21-acetate (V) was obtained.

Diacetate of 18-OH-DOC. (21,20-diacetoxy-18,20-epoxy-4-pregnen-3-one) (VII). When 18-OH-DOC was acetylated more drastically with 1:2 acetic anhydride:pyridine at 60° for 18 h there was obtained in addition to monoacetate (II) and the etiolactone of 18-OH-DOC, approximately 10% of a product which migrated as shown in Table 1 and analyzed as a diacetate (VII) ($^3H/^{14}C$ = 5·4, theoretical for a diacetate = 5·6). In contrast to the 21-monoacetate (II), this diacetate was stable on rechromatography. The Porter–Silber reaction was positive. Reduction of alkaline blue tetrazolium was slow, indicating that the second acetoxy group was at C-20 rather than at C-18 as suggested by Kahnt and co-workers [1] for their major acetylation product of 18-OH-DOC.

20-Methyl ketal of 18-OH-DOC-21-monoacetate. (21-acetoxy-20-methoxy-18,20-epoxy-4-pregnen-3-one) (V). In a typical synthesis, 0·5 mg 18-OH-DOC was treated with 5 ml of an alcohol containing 30 μl glacial acetic acid for 16 h at room temperature. Solvents were removed under vacuum with the aid of several additions of benzene. The residue containing the methyl ketal (III) was dissolved in 1 ml pyridine and acetylated with 0·2 ml acetic anhydride at room temperature for 16 h. Excess reagents were removed by evaporation and the acetylated derivative was isolated first by chromatography on system 4 (Table 1) and purified on a 1 cm diameter column containing 10 g neutral alumina. The column was developed with petroleum ether:benzene (4:1 v/v) and the product eluted with petroleum ether:benzene (1:4 v/v). Recrystallization from ethyl ether gave prisms with a corrected melting point on a Kofler block of 169–170°. The infrared spectrum (Fig. 5) showed carbonyl absorptions at 5·97 (conjugated ketone) and 5·77 (ester) μm but no hydroxyl group. There was no evidence of a C-20 ketone and the ester

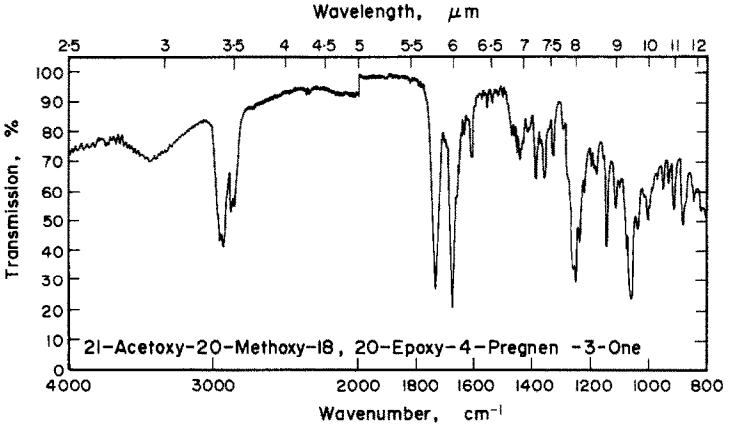


Fig. 5.

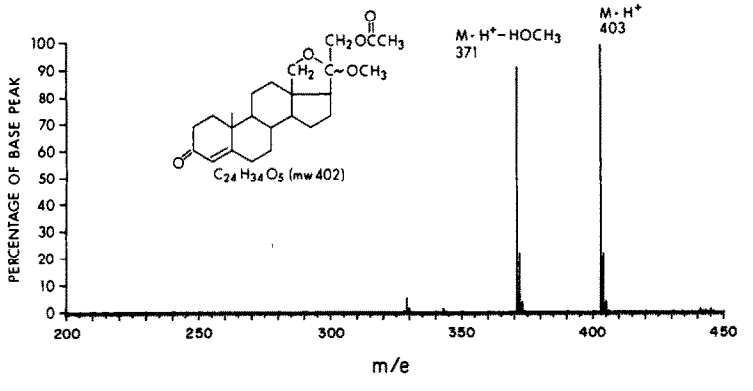


Fig. 6. Chemical ionization mass spectrum of methyl ketal-21-acetate derivative.

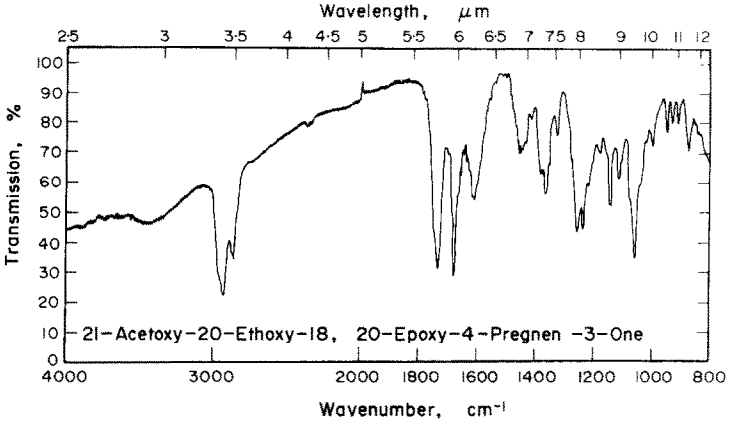


Fig. 7.

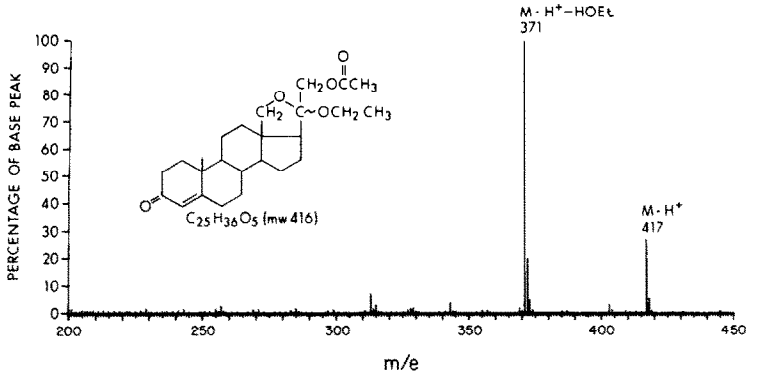


Fig. 8. Chemical ionization mass spectrum of ethyl ketal-21-monoacetate derivative.

band was not shifted to lower wavelengths as seen in the spectra of 21-acetoxy-20-ketones [23]. The chemical ionization mass spectrum (Fig. 6) showed a prominent protonated molecular ion and a major fragment at $m/e = 371$ due to loss of methanol.

20-Ethyl ketal of 18-OH-DOC-21-monoacetate. (VI) (21-acetoxy-20-ethoxy-18,20-epoxy-4-pregnen-3-one). The C-20 ethoxy derivative (VI) was prepared as described above for the methoxy derivative except that absolute ethanol was substituted for methanol. Chromatographic mobilities of the intermediate ethoxy derivative (IV) and its 21-acetate (VI) are shown in Table 1. A spectroscopically pure sample obtained after paper chromatography and elution from an alumina column showed carbonyl absorptions similar to those of the methyl ketal V in the infrared (Fig. 7) but differing in the fingerprint region. The chemical ionization mass spectrum (Fig. 8) showed a protonated molecular ion and a relatively larger major fragment at *m/e* 371 like that seen in the methyl ketal but due to loss of ethanol.

DISCUSSION

The formation of ketals occurs in two steps, of which the first involves the formation of a hemiketal by nucleophilic addition to the carbonyl group, and the second step proceeds by way of a carbonium ion intermediate whose formation determines the rate of the reaction. Since 18-OH-DOC is already in the hemiketal form, we are concerned with the mechanism of the second step. That this step should be acid-catalyzed is expected. Thus the reaction of 18-OH-DOC with methanolic or ethanolic acetic acid is not unusual. What was not expected, however, was the catalysis by trace impurities in reagent-grade redistilled alcoholic solvents. The acidic and proton-donating nature of these catalytic impurities is indicated by their removal from the alcohol by distillation from sodium hydroxide, or by the addition of an amine. The ease with which the reaction takes place, however, suggests, in addition, the participation of intramolecular factors. Two observations suggest a catalytic role for the C-21 hydroxyl hydrogen. One is that formation of the C-20 ketal occurs more readily with the 21-hydroxyl derivative than with the 21-acetate. The other is that the reverse reaction, removal of the C-20 ketal, which should proceed by way of the same transition state, occurs very readily when the 21-hydroxyl is free but not when it is acetylated. Further evidence for intramolecular factors in the unusual reactivity of the 18-OH-DOC structure is illustrated by the example of ketalization in the absence of external catalysis. When 18-OH-DOC-21-monoacetate was dissolved in methanol that had been distilled

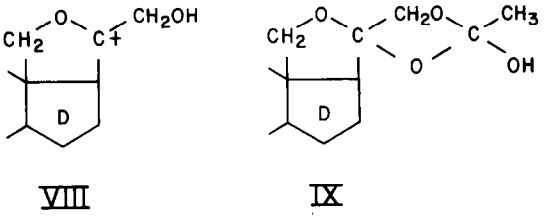


Fig. 9.

from sodium hydroxide, the only product was the C-20 methyl ketal of 18-OH-DOC. Removal of the acetate group was accompanied by equimolar ketalization. This suggests a mechanism in which the acetate carbonyl in some way facilitates the formation of the carbonium ion at C-20 (VIII), perhaps by way of an intermediate of the ortho ester type (IX), as shown in Fig. 9.

This description of the mechanism of one type of chemical alteration which 18-OH-DOC undergoes does not mean to imply that other reactions do not occur. Dimerization has been observed with 18-OH-DOC [24] and 18-OH-corticosterone [24] although we did not observe it under the conditions described here. Dimerization very likely occurs by the same mechanism as ketal formation except that the reactive intermediate carbonium ion at C-20 reacts with the C-21-alcohol of another molecule instead of the solvent.

The use of alcohols, which have generally been considered to be inert and safe for elution, dilution and storage of steroids must now be qualified. In addition, some discrepancies in the literature concerning the biological and spectroscopic properties [3] of 18-OH-DOC may now be interpretable in terms of variable content of ketal forms. With the recognition of the mechanism of formation of the two forms of 18-OH-DOC, however, the problem can be readily avoided in several ways. These include the use of alkali-distilled solvents, the addition of an amine, storage in the dry state, or the use of non-alcoholic solvents. Should the ketal forms already be present, the free steroid can be quantitatively regenerated by treatment with dilute mineral acid. It has also been possible to exploit the ease of selective formation and removal of these ketal groups to impart specificity to chromatographic purification procedures for this group of steroids (unpublished observations). The applicability of the reactions described here for 18-OH-20-keto-pregnanes generally, will be described in another report.

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