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MOLECULAR CONFIGURATION AND CONFORMATION OF ALDOSTERONE, 18-HYDROXY-11-DEOXYCORTICOSTERONE AND A NEW URINARY 18-HYDROXY-STEROID— AN N.M.R. STUDY

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SUMMARY

The ^{1}Hmr and ^{13}Cmr spectra of aldosterone in various solvents show only the tautomeric forms with hemi-acetal [11–18] and hemi-ketal [18–20] bridges.

The predominant form of 18-hydroxy-11-deoxycorticosterone (18-OH-DOC) in $CDCl_3$ (compound M of Dominguez) is the hemi-ketal [18–20] tautomer. Two forms are detected in CD_3OD: the hemi-ketal tautomer and very likely a dimer. Other derivatives are found in the presence of water and small amounts of acid. The 18-hydroxy-tautomer with a 20–C = O is not found.

Two unknown steroids (compound *x* and compound *y*) were isolated from the urine of patients with hypertension and/or adrenal disorders. The ^{1}Hmr spectroscopy indicates that the following structure can be proposed for compound *x*: *a*: a derivative of dihydro-18-hydroxy-deoxycorticosterone hydroxylated either at 4β and 9α or less probably at 5α and 9α. *b*: a derivative of dihydro-18-hydroxy-dehydrocorticosterone hydroxylated either at 4β or 5α or 9α. The behaviour of compound *x* in solution is very similar to that of 18-OH-DOC (predominance of the 18–20 hemi-ketal, formation of dimer and of other derivatives).

INTRODUCTION

The presence of an aldehyde or alcohol function at carbon 18 of corticosteroids implies the possible formation of hemi-acetal or hemi-ketal bridges between carbon 11 and 18 and/or 18 and 20 and thus the existence of tautomeric forms of these compounds.

A short time after the discovery of aldosterone, Simpson and Tait and their co-workers [1] demonstrated the existence of a hemi-acetal form (form 2 of Fig. 1). On the basis of i.r. spectrometry Ham *et al.* [2, 3] have postulated a third form of aldosterone (form 3 of Fig. 1). Similarly, Dominguez [4] demonstrated by paperchromatography the existence of two interconvertible forms of 18-hydroxy-11-deoxycorticosterone (18-OH-DOC), both for the synthetic compound and for the steroid isolated from biological material. These two forms were designated as M for the more polar form and L for the less polar form. Form M is the major component in chloroform while the less polar form L is produced in methanolic solution. In this solvent, the transformation to form L increases with dilution and time of standing until it becomes the major component in the mixture. Form L is also formed in formamide or pyridine solution but to a much lesser extent than in methanol. The two possible tautomeric forms of 18-OH-DOC are forms 4 and 5 (Fig. 2).

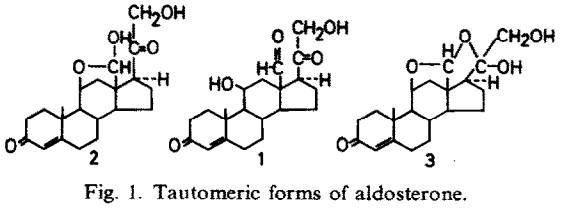

Fig. 1. Tautomeric forms of aldosterone.

Dominguez suggested that M is the 18–20 hemi-ketal form and L its corresponding methylether but the possibility that M is the 18-hydroxy-tautomer and L its corresponding 18–20 hemi-ketal cannot be entirely ruled out (Fig. 2). The hypothesis that M and L are respectively the two diastereoisomers of the 18–20 hemi-ketal form is highly improbable. Indeed, M and L differ for about 0·4 in R_F value while a difference in R_F value of about 0·05–0·10 is usually observed between diastereoisomeric steroids.

In 1970, Melby *et al.* [5] demonstrated that the tetrahydro-derivative of 18-OH-DOC is excreted abundantly in the urine of patients with different forms of hypertension. The authors believe that 18-OH-DOC, having a relatively low mineralocorticoid

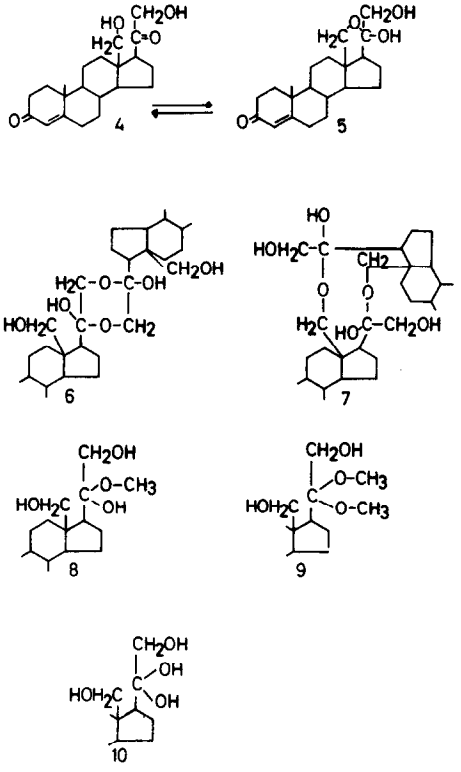

Fig. 2. Forms of 18-OH-DOC.

effect, is converted in such patients to a more active steroid in the same manner as 18-OH-corticosterone is transformed to aldosterone. In 1973, Dale and Melby [6] have isolated a steroid more polar than 18-OH-DOC from incubations of adrenals from patients with breast carcinoma. On the basis of their identification studies, the isolated compound was the 16α-hydroxy-derivative of 18-OH-DOC.

Adlercreutz *et al.* (1973) [7] have detected a new substance, called "hypersterone", in the urine of hypertensive patients. The mass spectrum of the oxidized "hypersterone" suggests a molecular structure similar to that of aldosterone with additional hydroxyl groups. However, the mass spectrum of the parent compound was not obtained.

Up to now no stimulatory effect of these new compounds on active sodium transport has been demonstrated.

Aldosterone and 18-OH-DOC were estimated in our laboratory in the urine of 404 patients most of them with various forms of hypertension (labile hypertension, fixed hypertension, hypertension with vascular and renal disorders, malignant hypertension, renal hypertension, primary aldosteronism) and adrenal disorders. Both steroids were estimated by gas chromatography of the γ-lactone obtained after periodic acid oxidation of the isolated compounds. In the urine of 65 patients variable amounts of 18-OH-DOC and/or of two unknown compounds, called *x* and *y* (both more polar than aldosterone) were detected. Compound *x* and compound *y* were found in 36 cases and 18-OH-DOC in 29 cases (Table 1). Generally, compound *x* and/or *y* were not found at the onset but after evolution of the disease. Plasma potassium is low in the patients with large amounts of *x*.

Very large amounts of compound *x* were found in the urine of a woman (56 years) with hypokalemia, sodium retention, cyclic oedema, moderate hypertension and low P.R.A. Important amounts of compound *x* and small amounts of compound *y* were found in the urine of a second patient (a woman of 42 years) with fixed hypertension and hypokalemia.

The urine of the two patients was collected during 40 days. Compound *x* and *y* were isolated from the pooled urine.

In this paper we wish to represent our n.m.r. studies on the molecular configuration and conformation of aldosterone, 18-hydroxy-deoxycorticosterone and the unknown compound *x*.

EXPERIMENTAL

a. *Nuclear magnetic resonance.* The n.m.r. spectra of the compounds studied were measured in $CDCl_3$, DMSO-d_6, CD_3OD and D_2O.

The ^1H and ^{13}Cmr spectra were performed on a Varian H.A.-100 and a HFX-90 Bruker spectrometer.

b. *Synthetic steroids.* Aldosterone was furnished by Ciba (Basle) and 18-OH-DOC by Searle (Mexico).

c. *Isolation procedure for compounds* x *and* y. The isolation procedure is as follows. The urine is hydrolyzed and extracted with dichloromethane. The dichloromethane extract is washed with NaOH 0·1 N and evaporated to dryness. The residue is partitioned between benzene–water. The water phase is extracted with chloroform the extract evaporated to dryness and the residue chromatographed on a celite column using the Bush-system B_4. The fraction from 45 to 85 ml is collected and evaporated to dryness. The residue is chromatographed on a Sephadex LH 20 column using benzene–dichloromethane–methanol (60:35:5 by vol.) as elution liquid. The fraction from 16 to 200 ml was collected, evaporated to dryness and the residue chromatographed on paper in the system benzene–acetone–water (2:1:2). The zone between aldosterone and 6β-hydroxycortisone was cut out

Table 1. Distribution of 18-OH-DOC, compound *x* and compound *y* in 404 patients

18-OH-DOC alone : 11	*y* alone : 10
18-OH-DOC + *x* : 4	*x* + *y* : 5
18-OH-DOC + *y* : 0	18-OH-DOC + increased aldosterone : 8 on 11 cases
18-OH-DOC + *x* + *y* : 1	*x* + aldo : 3 on 9 cases
x alone : 9	*y* + aldo : 7 on 10 cases

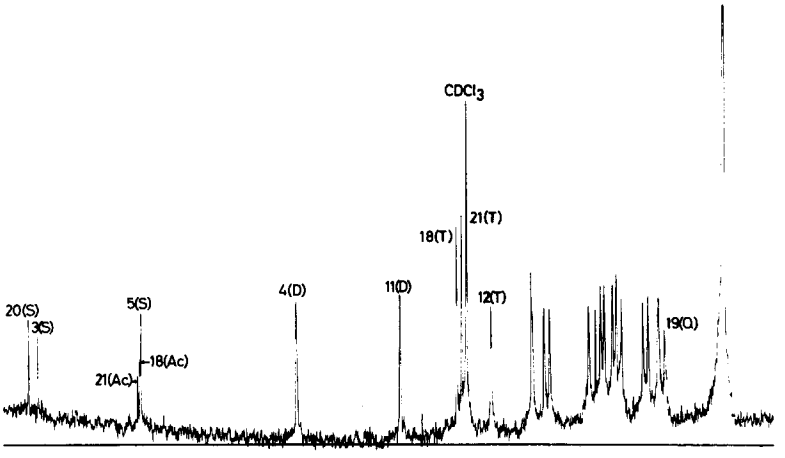

Fig. 3. ^{13}Cmr spectrum of aldosterone-diacetate in $CDCl_3$ at 22·63 MHz.

from the chromatogram and eluted with ethanol. The periodic acid oxidation was carried out on a small volume of the eluate of the paper chromatogram zone followed by gas chromatography. Here again, a peak was observed with the same retention times as those found for compound *x* or *y* during routine estimation of aldosterone. The identification studies were carried out on the ethanolic extract. No maximum at 380 nm was seen in the absorption spectrum after the isonicotinic acid hydrazide reaction [8] indicating the absence of a Δ^4-3-keto group.

RESULTS AND DISCUSSION

In previous works [9, 10] the ^{1}Hmr spectrum of aldosterone and 18-OH-DOC has been studied in various solvents. Recently, the ^{13}Cmr spectra of aldosterone, its acetylated derivatives and of 18-OH-DOC with both the broad band and the off resonance method have been performed.

The results of these experiments can be summarized as follows:

1. *Aldosterone and acetylated derivatives*

a. ^{1}*Hmr spectra.* The solutions of d-aldosterone in $CDCl_3$, DMSO-d_6, C_5D_5N, CD_3OD and D_2O at 25°C contain only a mixture of the tautomeric forms 2 and 3 (Fig. 1) in the same proportion. In CD_3OD and in D_2O, the concentration of 2 increases with temperature. A molecule of water is bound to the hydroxyl group at carbon 21. The aldehyde form was not found. In a solution of aldosterone-21-acetate the forms 2 and 3 were found in the proportion 1 to 3. Aldosterone diacetate only exists in one form, most likely form 2 (Fig. 1).

b. ^{13}*Cmr spectra.* The ^{13}Cmr spectra (Fig. 3) confirm that the solutions of aldosterone and its monoacetate in $CDCl_3$ only contain forms 2 and 3. The spectrum of the diacetate in $CDCl_3$ shows 25 carbon resonances, four of which being carbonyl carbons (carbon 3, carbon 20 and the two carbons of acetate) indicating that only the form 2 is detected.

2. 18-*Hydroxy*-11-*deoxycorticosterone*

It was of interest to perform the spectrum in both $CDCl_3$, in which the major component is the compound M of Dominguez, and in CD_3OD which induces the formation of compound L. However, the spectrum in DMSO-d_6 was very useful to achieve the attribution of the lines. Finally, the ^{1}Hmr spectrum in D_2O was recorded.

a. ^{1}*Hmr spectrum in* $CDCl_3$ (Fig. 4). A strong singlet (2 protons) and a quartet (2 protons) are found respectively at 3·80 ppm and at 3·70 ppm, corresponding to the resonances of the methylene protons at carbon 18 and carbon 21. The quartet should be an AB pattern with Jgem = |11 Hz|. The fact that in aldosterone the 11α proton, which is bounded to a carbon atom bearing a hemi-ketal oxygen, is found at a lower field than the CH_2 group at carbon 21 allows us to attribute the peak at 3·80 ppm to the CH_2 group at carbon 18. Two hydroxyl protons are detected.

It is impossible to determine the predominant form of 18-OH-DOC in $CDCl_3$ only on the basis of its ^{1}Hmr spectrum. However, the fact that the ^{1}Hmr spectrum of 18-OH-DOC in $CDCl_3$ does not change after 50 days indicates the presence of the same

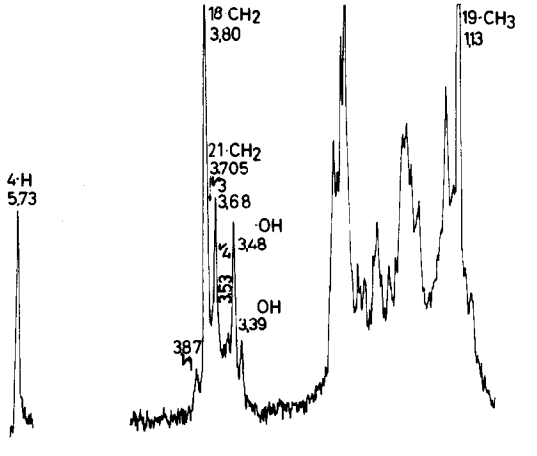

Fig. 4. ^{1}Hmr spectrum of 18-OH-DOC in $CDCl_3$ at 100 MHz.

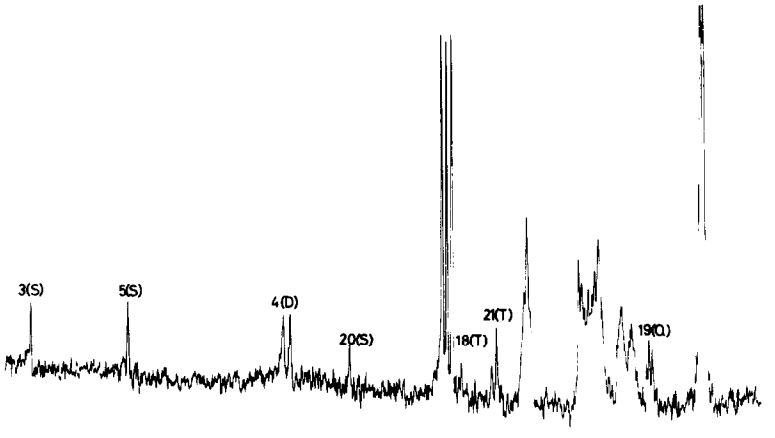

Fig. 5. ^{13}Cmr spectrum of 18-OH-DOC in $CDCl_3$ at 22·63 MHz (off resonance).

predominant form as in the crystalline compounds, namely form 5 (Fig. 2) as no absorption band at 1700 cm.$^{-1}$, characteristic for a ketone at carbon 20 of a pregnane derivative, is found during i.r. spectrometry. The formation of a dimer (Fig. 2, 6, 7) may be rejected. Indeed the molecular weight measurement made by vapor-phase osmometry* in $CDCl_3$ gives 382 for 18-OH-DOC (mol. wt. = 343). The diastereoisomer at carbon 20 could not be obtained.

Otherwise, in 13 steroids with a —CO—CH_2OH moiety at carbon atom 17 [10] the resonance of the methylene protons at carbon atom 21 are found at about 4·10 to 4·60 ppm (Jgem = |19 Hz|). In form 3 of aldosterone, which has no carbonyl group at carbon 20, the shift and Jgem are respectively 3·5 ppm and |11 Hz|. The 21-CH_2 group of 18-OH-DOC resonate at 3·70 ppm. However the methylene protons could lie in the shielding zone of the carbonyl. A ^{13}Cmr spectrum will be more instructuve.

b. *^{13}Cmr spectrum in $CDCl_3$.* The ^{13}Cmr spectrum undoubtedly confirms that the molecular configuration of form 5 is the predominant form of 18-OH-DOC in chloroform. Indeed, 21 carbon lines are detected in the proton broad band decoupling spectrum indicating one predominant form of 18-OH-DOC. The spectrum shows only one resonance line in the carbonyl region corresponding to the carbonyl group at carbon 3 of steroids with a Δ^4-3-keto group ($\simeq$197 ppm for TMS) [10, 11]. No peak is found at about 210 ppm corresponding to the carbonyl group at carbon 20 of steroids with a —CO–CH_2OH side chain at carbon 17. A downfield shift of the carbon atoms 18, 20 and 21, which are bearing an oxygen atom, may also be expected. The off resonance spectrum (Fig. 5) indicates that the triplets at 71·6 and 61·5 ppm are the peaks of the carbon atoms 18 and 21. Thus, the singlet at 103·9 ppm is the line of carbon atom 20 bonded to two oxygen atoms.

c. *^{1}Hmr spectrum in DMSO-d_6.* The spectrum of 18-OH-DOC in DMSO-d_6 recorded immediately after dissolving the compound in this solvent (Fig. 6a) indicates the presence of two forms. Indeed, the

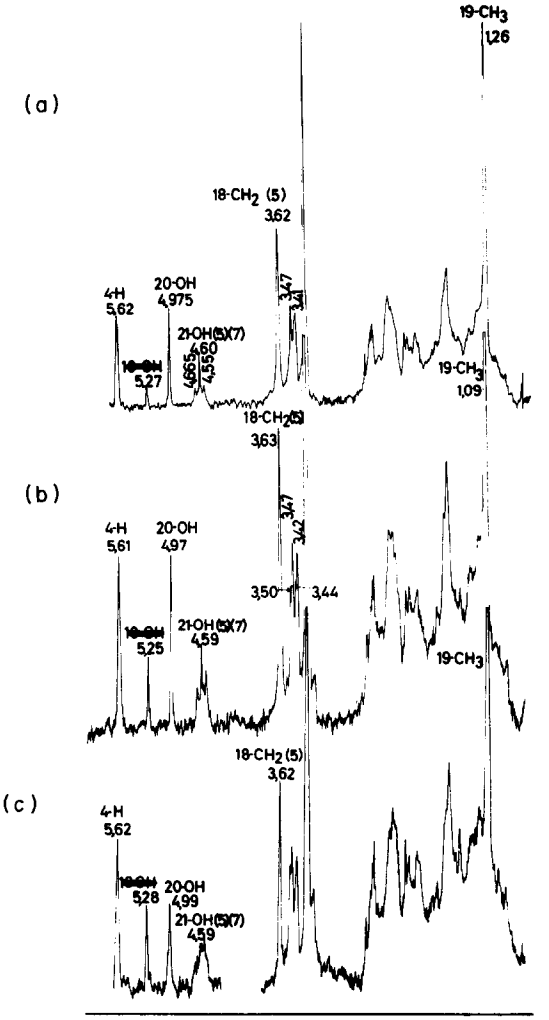

Fig. 6. ^{1}Hmr spectrum at 100 MHz of 18-OH-DOC in DMSO-d_6. a. Immediately after dissolving the compound; b. 4 hours after dissolving the compound; c. Four days after dissolving the compound.

* The measure was performed on a "Dampfdruck Osmometer Knauer".

line at 5·27 ppm, which certainly corresponds to one proton, is much smaller than the signal of the proton at carbon 4 of 18-OH-DOC. The area of the peak at 3·62 ppm, which corresponds to two protons and is analogous to the peak at 3·80 ppm in $CDCl_3$, is not twice the peak area corresponding to the proton attached at carbon atom 4.

The singlets at 5·25 ppm and 4·97 ppm and the multiplet centered at 4·60 ppm are hydroxyl protons shifted to higher field by increasing the temperature. The spectra recorded after 4 h and 4 days (Fig. 6b and 6c) show a progressive decrease of the lines at 4·97 and 3·63 ppm and an increase of the line at 5·25 ppm. The shape of the peak at 5·60 ppm is modified but its area remains unchanged.

Immediately after the preparation of the solution two peaks are found at 3·41 ppm and at 3·47 ppm. Four h later (Fig. 6b) the spectrum in this region shows four lines at 3·50 ppm, 3·47 ppm, 3·44 ppm and 3·42 ppm, which partially overlap each other. Their shifts are not modified by variation of the temperature. Four days later an increase of the signal of the first and the fourth line and a decrease of the signal of the second and the third line are observed.

After irradiation at the frequency of the multiplet centered at 4·60 ppm, the line at 3·50 ppm remains unchanged; however, in the place of the peaks at 3·47 ppm, 3·42 ppm and 3·41 ppm a large line at 3·44 ppm and a smaller line at 3·36 ppm are found. Otherwise, irradiation at the frequency of the line at 3·47 ppm modifies the signal at 4·60 ppm.

Form 5 (Fig. 2) being predominant in the solid state, most likely remains more abundant in the DMSO solution and the lines whose area is decreasing with time should correspond to that isomer. On the contrary, one which increases with time, should be attributed to another form.

The doublet at 3·41 ppm to 3·47 ppm and the singlet at 3·62 ppm are the resonances of the methylene groups at carbon 21 and carbon 18; they correspond respectively to the quartet at 3·70 ppm and to the singlet at 3·80 ppm of the ^{1}Hmr spectrum in $CDCl_3$. These resonances are decreasing with time and thus should belong to form 5. This is also an indication that the isomer detected in $CDCl_3$ is form 5.

The fact that the resonance of the methylene group at carbon 21 is a quartet in $CDCl_3$ and a doublet in DMSO can be explained as follows. Hydrogen bonds most likely exist in $CDCl_3$ between the hydroxyl group at carbon 21 and the hydroxyl group at carbon 18 or the hemi-ketal oxygen. Such bonds do not exist in DMSO-d_6 so that the methylene group at carbon 18 or carbon 21 is reoriented in such a manner that the repulsion forces between the three oxygen atoms at carbon 18, 20 and 21 are reduced. Thus, the magnetic environment of the methylene group at carbon 21 is modified in such a manner that a quasi magnetic equivalence for these two protons is obtained.

The lines at 3·50 and at 3·36 ppm are the resonances of the methylene groups at carbon 18 and carbon 21 in the other form. It is impossible to attribute these lines. The singlet at 4·99 ppm, which decreases with time of standing, corresponds to the hydroxyl proton at carbon 20.

d. *^{13}Cmr spectrum in DMSO-d_6* (broad band proton decoupling). More than 21 carbon lines are detected (Fig. 7) indicating the presence of two forms. Otherwise, no resonance corresponding to a carbonyl group at carbon 20 is seen while a peak at almost the same frequency of carbon atom 20 in $CDCl_3$ (form 5, Fig. 2) is observed. A small peak exists at a somewhat higher field. These facts indicate that the major form detected in DMSO-d_6 is 5 (Fig. 2). The second form should be a dimer (forms 6 or 7 of Fig. 2). In view of the symmetry of the two conjugated molecules in the dimer it may be admitted that the symmetrical protons and carbons of each molecule are magnetically equivalent and resonate at the same frequency. Otherwise, the resonance frequency of the methylene protons at carbon 18 and at carbon 21 and of the 20-^{13}C should not be very different in form 5 and form 7. The CH_2 group at carbon 21 in form 6 should be more shielded, and the CH_2 group at carbon 18 somewhat more deshielded than

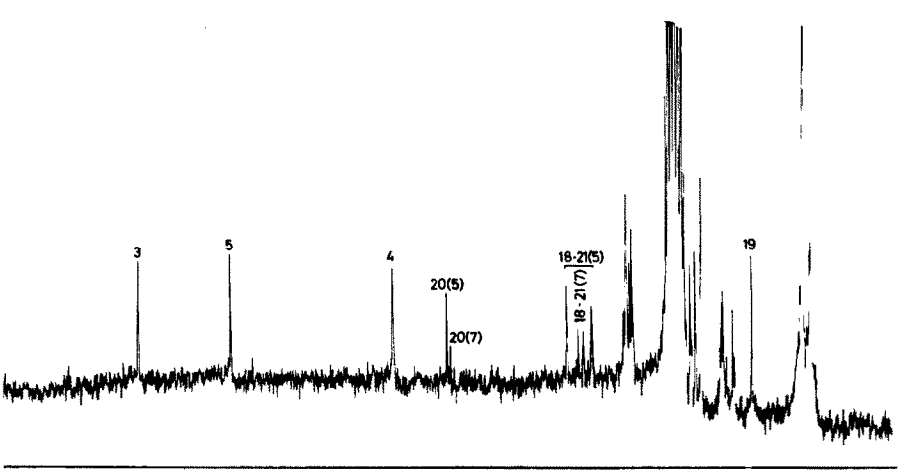

Fig. 7. ^{13}Cmr spectrum at 22·63 MHz of 18-OH-DOC in DMSO-d_6 (broad band proton decoupling)

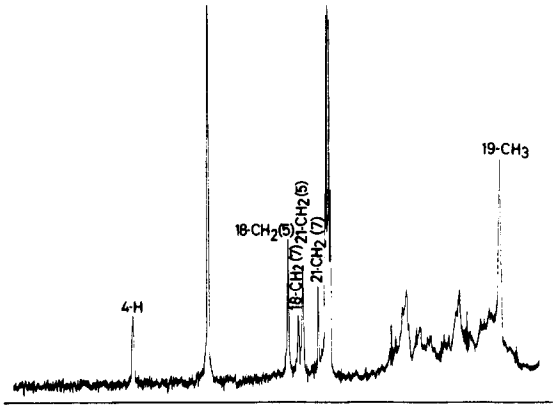

Fig. 8. ^{1}Hmr spectrum of 18-OH-DOC in CD_3OD containing no water at 100 MHz.

in form 5. It may be suggested that form 6 is the second component found in DMSO.

e. *^{1}Hmr spectrum in CD_3OD.* The ^{1}Hmr spectrum (Fig. 8) in pure CD_3OD, containing no or only very small amounts of water, is similar to that in DMSO-d_6. It shows resonance peaks at 5·70 ppm and at 1·15 ppm, corresponding respectively to the proton at carbon 4 and to the methyl group of carbon 19, and four signals (3 singlets and 1 doublet) respectively at 3·79, 3·66, 3·60 (D) and 3·41 ppm (Fig. 8) in the middle region of the spectrum. The doublet probably constitutes the inner lines of a quartet (AB system), the outer lines being small and not detected.

Each of the 4 signals represents two protons. The peaks at 3·79 ppm and at 3·60 ppm correspond respectively to the CH_2 group of carbon 18 and 21 of form 5 in $CDCl_3$ and DMSO. Their respective area is about twice the area of the signal at 3·66 ppm or 3·41 ppm which probably correspond to the CH_2 group of carbon 18 and 21 respectively in another form (7 or more probably 6). The spectrum is not changed either by a conservation of 20 days in CD_3OD or by an increase of the temperature from 25°C to 45°C. The ^{1}Hmr spectrum in $CDCl_3$ recorded after evaporation of the deuterated methanol is very similar to that of 18-OH-DOC in $CDCl_3$ without action of CD_3OD. Moreover, no absorption band at 1700 cm.$^{-1}$, characteristic of a ketone at carbon 20, is found during i.r. spectrometry of the solution of 18-OH-DOC in CD_3OD (Beckman i.r. 12 spectrophotometer).

The ^{1}Hmr spectrum of 18-OH-DOC in CD_3OD containing small amounts of water (pH = 4) and its evolution with time is quite different from that in pure CD_3OD (Fig. 9a) Immediately after dissolution of the compound the same resonance peaks, observed in the spectrum with pure CD_3OD, are seen; the peaks at 3·79, 3·66 and 3·41 ppm decrease with time while the peak at 3·60 ppm increases with time. Moreover, a series of resonance lines at 3·85, 3·73, 3·57, 3·51 (doublet) and 3·48 ppm, which are not found in pure CD_3OD or other solvents, appear and increase with time. Another peak at 3·30 ppm is the result of an overlapping of an increasing peak and a decreasing peak at about the same frequency. The lines at 3·79, 3·66, 3·60 and 3·41 ppm may correspond to the CH_2 group of carbon atoms 18 and 21 of the form 5 and 6 (or 7) and decrease with time. The other lines which are also in the frequency region of the CH_2 groups at carbon atoms 18 and 21, correspond to one or more supplementary forms increasing with time and probably constitute two overlapping AB systems. An equilibrium is reached after 20 days (Fig. 9b). Furthermore, a group of lines increasing with time occurs at 4·15 ppm. After evaporation of

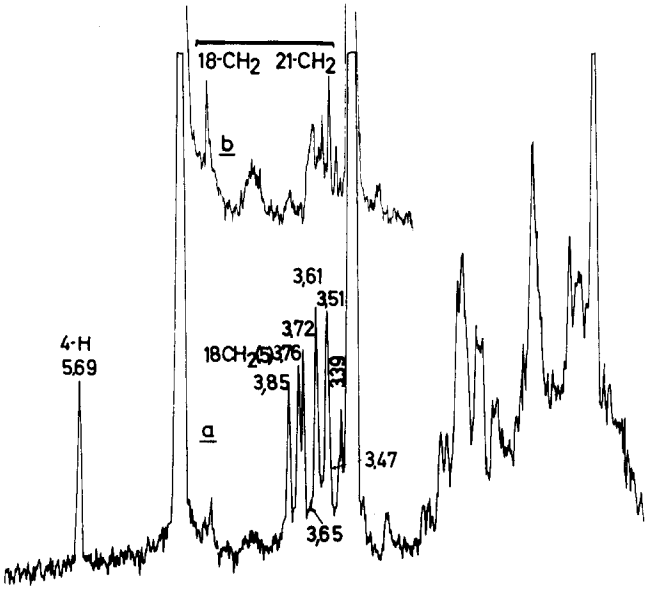

Fig. 9. ^{1}Hmr spectrum of 18-OH-DOC at 100 MHz in CD_3OD containing water pH = 4. a. 12 days after dissolving in CD_3OD; b. 20 days after dissolving in CD_3OD.

the deuterated methanol and resolving in $CDCl_3$ (Fig. 13a) a ^{1}Hmr spectrum similar to that in CD_3OD and with a better resolution of the peaks is recorded. The multiplet at about 4·16 ppm probably results from an overlapping of two quartets. These peaks are not seen neither in the $CDCl_3$ nor in the DMSO-d_6 spectrum. These quartets are probably the resonances of the CH_2 group of carbon atom 21 of form 8, 9 and/or 10.

It may be proposed that 18-OH-DOC reacts with CD_3OD containing small amounts of water and acid resulting in the formation of a hemi-acetal and/or acetal and/or 20-hydroxy-derivatives at carbon atom 20 (Fig. 2, forms 8, 9, 10). The two quartets at 4·16 ppm should be the resonances of the methylene protons at carbon atom 21 in form 8 and 9. This correspond with the observations of Bhacca *et al.* who have found a chemical shift of 4·15 ppm for the 2α-proton of 3,3'-dimethoxy-2β,19-epoxy-5α-androstan-17β-ol [11]. The magnetic environment of this proton and of the protons of carbon atom 21 in form 8 and 9 are similar. A reaction of CD_3OD with the carbonyl group at carbon atom 3 may be considered as very improbable since this would induce a modification of the resonance frequencies of the protons at carbon atoms 4 and 19. An indication of the presence of methoxy groups at carbon atom 20 can be obtained by evaporating a solution of 18-OH-DOC in CH_3OD containing small amounts of water and resolving the residue in $CDCl_3$. The ^{1}Hmr spectrum in $CDCl_3$ recorded after this CH_3OD treatment shows resonance peaks at 2·26, 2·44 and 2·56 ppm which probably correspond to the protons of three methoxy groups. The other regions of the spectrum are unchanged.

f. *^{13}Cmr spectrum in CD_3OD without water.* That spectrum (Fig. 10) is almost identical to that recorded in DMSO (Fig. 7). Two forms are present (5 and 6 or 7). No signal for a carbonyl at carbon 20 is found.

g. *Spectrum in D_2O.* In spite of the poor solubility of the steroid in water it was possible to measure the n.m.r. spectrum in D_2O (pH = 4). In these conditions a spectrum (Fig. 11) similar to that in CD_3OD with a small amount of water has been recorded. Indeed, the lines of the protons at carbon atoms 4 and 19 are found at analogous frequencies while the protons of the methylene groups of carbon 18 and 21 are situated between 3·00 and 4·50 ppm.

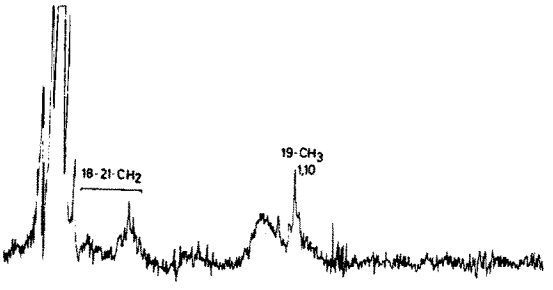

Fig. 11. ^{1}Hmr spectrum of 18-OH-DOC in D_2O (pH = 4) at 90 MHz—Fourier transform—80000 scans.

3. *Identification study of compound* x *by ^{1}Hmr*

The results of the n.m.r. spectra can be summarized as follows. The spectrum of compound *x* in $CDCl_3$ (Fig. 12) is consistent with that of about 750 μg (first patient) or 75 μg (second patient) of a steroid with a molecular weight near 350. These amounts correspond approximately to the amount of compound *x* found by gas chromatography in the urine of both patients. The spectrum shows a sharp line at 1·31 ppm which corresponds to a methyl group protruding above a methylene envelope, and two "humps" respectively at about 3·65 ppm and 4·00 ppm. It is very similar in the two cases.

Compound *x* was deuterated with CD_3OD. The methanol was evaporated to dryness and the spectrum of the deuterated compound was performed in $CDCl_3$.

A comparison of the spectrum of this deuterated compound with that of deuterated 18-OH-DOC is

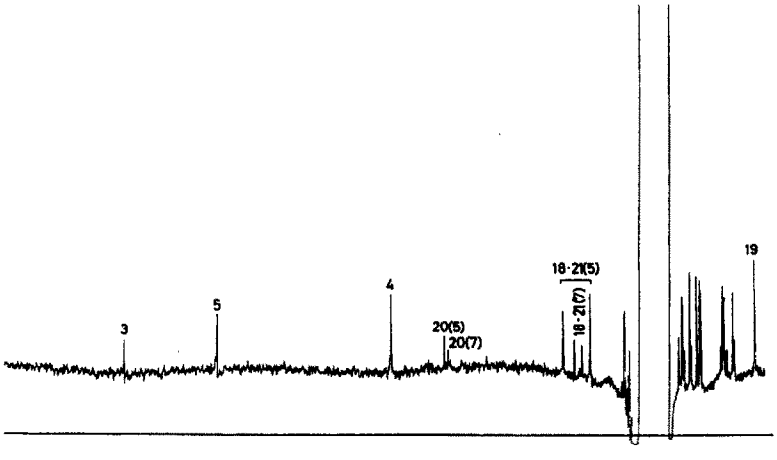

Fig. 10. ^{13}Cmr spectrum of 18-OH-DOC in CD_3OD without water at 22·63 MHz (broad band proton decoupling).

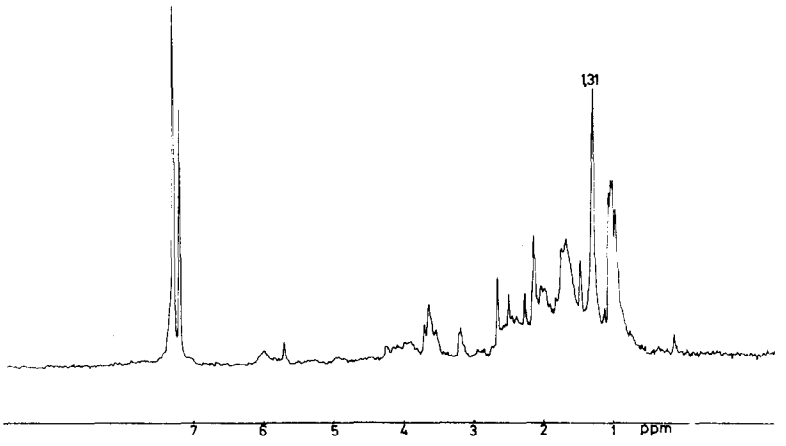

Fig. 12. ^{1}Hmr spectrum of compound x in $CDCl_3$ at 90 MHz—Fourier transform—2340 scans.

very instructive. Between 3·40 and 4·00 ppm, the spectrum is almost identical to that of 18-OH-DOC indicating the same molecular configuration of rings C and D, the 18-OH group and the 17-side chain (Fig. 13). However, at the other ranges of frequencies, the spectra of 18-OH-DOC and x show important differences.

1. The 19-CH_3 is shifted downfield (1·11 ppm in 18-OH-DOC, 1·32 in x).

2. The spectrum of compound x contains no lines in the olefinic proton range of frequency. The area of the peak at 5·83 is small. It does not belong to x.

3. The methylene envelopes of deuterated 18-OH-DOC and x are different.

The following preliminary conclusions can be drawn from these observations:

1. Compound x has no Δ^4-3-keto group. The removal of the double bond between carbon 4 and carbon 5 and the complete reduction of the carbonyl group at carbon 3 must result in an upfield shift of the protons at carbon 19 of respectively 0·25 ppm and 0·42 ppm [13] (14).

2. Compound x is probably a 5α-steroid. Indeed, a steroid with 5β conformation would result in a downfield shift of the protons at carbon 21 of about 0·40 ppm as in the case of 5β-tetrahydrocortisol [10].

3. The changes in the methylene envelope observed after deuteration confirms the presence of supplementary hydroxyl groups. Protons substituted by those polar groups are removed or shifted out of the methylene envelope. Furthermore, the hydroxyl protons of a secondary alcohol function (f.i. the hydroxyl function at carbon 11 of corticosterone) often resonate at these frequencies [10].

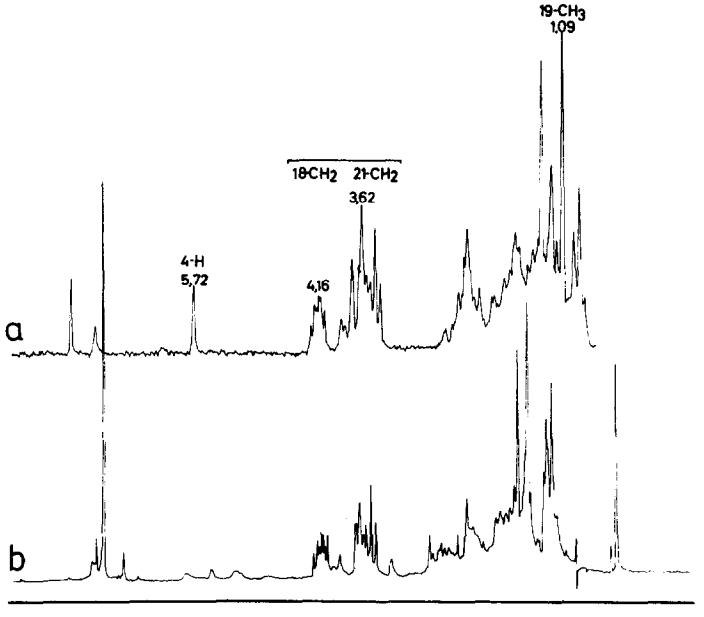

Fig. 13. ^{1}Hmr spectrum of (a) 18-OH-DOC at 90 MHz after action of CD_3OD during 20 days, evaporated and resolving in $CDCl_3$; (b) Compound x at 90 MHz in $CDCl_3$ after deuteriation by CD_3OD—Fourier transform—16890 scans.

4. Compound x can have no more than one secondary alcohol function. A proton, which is bound to the same carbon atom as the hydroxyl group (e.g. the 11α-proton of corticosterone), usually resonates at about 3·40 to 4·00 ppm [10, 12, 14]. Such a proton is coupled with 2, 3 or 4 neighbouring protons and constitutes a multiplet of low amplitude. However, more than one such proton would modify the spectrum between 3·40 and 4·00 ppm.

5. The fact that the spectrum of compound x compared to that of 18-OH-DOC shows both the downfield shift of the protons at carbon 19 and the same shift of the protons at carbon 18 and carbon 21 indicates that the hydroxyl groups are probably bound to a carbon ring A or B. Moreover, the R_F value observed during paper chromatography also suggests a polarity consistent with a substitution of ring A or B.

6. Bearing in mind that the hydroxyl groups should induce a downfield shift of the protons at carbon 19 of about 0·40 to 0·50 ppm, what can be the position of the hydroxyl functions on ring A or B? The data from the literature [13, 14] indicate that the hydroxyl groups at 2β, 4β, 5α, 6β and 9α alone can induce a downfield shift of respectively 0·26, 0·24, 0·18, 0·23, 0·19 ppm in 5α steroids*. An alcohol function at 9α may not be excluded since a 9α hydroxyl generally does not modify the resonance of the protons at carbon 18 and carbon 21 [14, 15]. A hydroxyl function at carbon 6 does not correspond with the R_F value of compound x observed during paper chromatography. A hydroxyl group on carbon 2 is no more probable since a 2-hydroxylated pregnane derivative has not been isolated from biological fluids. The probability of a double hydroxylation at carbon atoms 4 and 9 is greater than one at 5 and 9 as a hydroxyl function at 5 and 9 only induces a downfield shift of 0·19 ppm of the methyl protons [13, 14].

7. Compound x has probably a carbonyl group at carbon atom 3. A hydroxyl group at carbon 3 usually causes a very small shift of the protons at carbon 19 [13, 14]. However, a hydroxyl group at carbon 3 together with other hydroxyl groups would considerably modify the spectrum between 3·40 and 4·00 ppm, and the removal of the whole Δ^4-3-keto group would induce a downfield shift of 0·18 ppm for the proton at carbon 18. A urinary dihydroderivative of corticosteroids is not common. However small amounts of 5β-dihydroaldosterone are found in human urine [15].

8. From the considerations mentioned in 6 and 7 it may be deduced that compound x is a steroid without a C=C double bond in ring A with probably a carbonyl group at carbon 3 and two hydroxyl functions either at carbon 4 and carbon 9 or at carbon 5 and carbon 9.

* Downfield shifts of 0·12 ppm, 0·00 ppm and 0·24 ppm for a hydroxyl group respectively at 1β, 6α and 6β are found in the literature for 5β-steroids [13, 14].

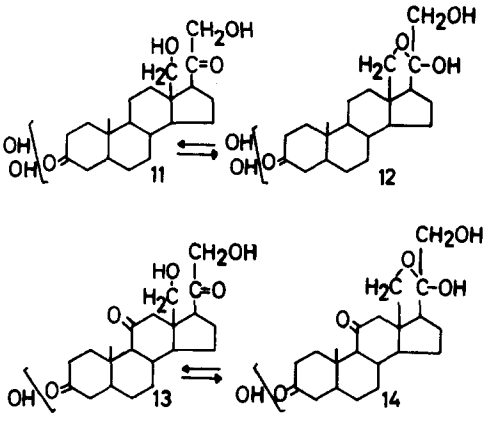

Fig. 14. Molecular configurations proposed for x.

Dihydro-18-OH-11-dehydrocorticosterone hydroxylated at carbon 4, carbon 5 or carbon 9 (Fig. 14) can also give a chemical shift of 1·32 ppm of the proton at carbon 19. However, in this case the proteins at carbon 18 and carbon 21 should lie on the surface of the shielding cone of the carbonyl group at carbon 11 as in the case of 11-dehydrocorticosterone [10].

9. Our studies indicate that the following structures of compound x can be proposed (Fig. 14):

a: a derivative of dihydro-18-hydroxy-deoxycorticosterone hydroxylated either at 4β and 9α or at 5α and 9α.

b: a derivative of dihydro-18-hydroxy-dehydrocorticosterone hydroxylated either at 4β or 5α or 9α.

10. It is likely that the same forms exist as for 18-OH-DOC in water and in methanol with small amounts of water and acid (form 12 of Fig. 14 and analogous of 6–10 of Fig. 2). The form 11 is not detected. The peaks between 2·26 and 2·52 ppm are probably methoxy signals at the same frequencies as for 18-OH-DOC in CH_3OD. Indeed compound x probably reacts with water and with methanol during the isolation, especially during chromatography on the Sephadex LH-20 column.

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