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LESS POLAR FORMS AND DERIVATIVES OF 18 HYDROXY-CORTICOSTERONE

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SUMMARY

The formation of various compounds upon reaction of 18 hydroxycorticosterone with neutral and acid-added alcohols as well as with acidic aqueous solutions is described. The time course of their formation in such media has been studied. Some of these compounds have been isolated and tentatively identified by paper chromatography employing double isotope techniques and by mass spectrometry. The more polar form has a C-20 hemiketalic structure; the single less polar form obtained upon storage in neutral methanol would be the methylketal at C-20 but the less polar fraction having the same mobility in the chromatographic system employed, obtained upon reaction with acidic aqueous solutions, seems to be formed by two different dehydration products.

INTRODUCTION

Eighteen-hydroxylated pregnane derivatives, upon storage in certain media, are spontaneously converted into less polar compounds [1, 2]. The conversions take place when the more polar forms of these 18-hydroxylated steroids are dissolved in acidic-aqueous solutions [3, 4] or dissolved and stored in alcohols [1, 2, 5, 7] or other organic solvents [2, 5]. A considerable amount of research has been carried out on this subject, ranging from conversion studies [2, 5, 6] to structure determinations by physical methods [8, 9]. These studies have occasionally led to contradictory results admitting a variety of possibilities for the less polar structures (see DISCUSSION).

Much of the confusion in this field might have been due to the lack of a systematic investigation on all less polar forms originated from the more polar ones in different media. Here we report the first attempt to achieve this goal, taking as a model one of the more hydroxylated, naturally occurring compounds—18 hydroxycorticosterone—whose possible importance as a hormone has been discussed [10], and whose precursor ability has been repeatedly sustained. Unexpectedly, 18 hydroxycorticosterone yielded, under certain experimental conditions, many of these less polar forms. Hence, for the sake of clarity, each isolated fraction is characterized by its relative mobility to the parent compound in the Bush B_5 system [11]. This more polar parent compound is here, as is usual [2], denominated M. The generic symbol L, implying one single less polar form, has been replaced by seven symbols. R_M 1.84, R_M 5.26, R_M 6.34, R_M 7.18 represent fractions with these mobilities obtained in acidic alcoholic and/or acidic aqueous solutions (see RESULTS). R_M 4.33, R_M^* 4.33 and R_M^{**} 4.33 designate fractions with this mobility obtained, respectively, upon reaction in neutral methanol, acidic aqueous and acidic alcoholic solutions.

Radioinert and radioactive material responding to the characteristics of the forms of different polarity was obtained under various, strictly controlled conditions and then M, R_M 4.33 and R_M^* 4.33 were analysed for their structures. The time course of formation of these three compounds, as well as of all others, was studied.

MATERIAL AND METHODS

Steroids. 18 Hydroxycorticosterone (18-OH-B) was purchased from Ikapharm, Ramat-Gan, Israel. [1, 2-^{3}H]-18-OH-B was biosynthesised as previously described [5]. [4-^{14}C]-18-OH-B was obtained in a similar fashion.

Solvents. AR absolute methanol was purchased from Merck, Darmstadt and was rectified in the laboratory. Absolute ethanol and *n*-butanol were AR from Carlo Erba, Milano and were rectified. Twice glass-distilled water was used. AR hydrochloric acid was from Merck. ^3H_2O and $^{14}CH_3OH$ were generous gifts from the Comisión Nacional de Energía Atómica, Argentina.

Chromatography and measurement of radioactivity. Paper chromatograms were developed in the Bush B_5 system [11]. Radioactive steroids were detected on paper strips by means of a Packard Radiochromatogram Scanner model 7201. Radioinert spots were located by quenching of paper fluorescence employing

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a Mineralight short wave U.V. S-11 lamp. Radioactivity was measured in a Packard Liquid Scintillation Spectrometer model 3003. The scintillation solution contained 3 g PPO and 100 mg dimethyl POPOP per liter of toluene. For double isotope counting, a channel ratio method employing three channels, was used.

Mass spectrometry. Electron impact spectra were obtained on a Varian-Mat CH-7 mass spectrometer, provided with a direct-insertion probe. Conditions are indicated for each case.

RESULTS

Transformations of the more polar (M) form of 18-OH-B into the less polar ones was performed in: (a) anhydrous neutral methanol; (b) aqueous neutral methanol; (c) anhydrous neutral ethanol and *n*-butanol; (d) 10^{-2} N HCl in water; (e) 10^{-2} N HCl in methanol, ethanol and *n*-butanol. In these media the time course of appearance of each less polar form was studied. Furthermore R_M 4.33 and R_M^* 4.33, as well as M, were analysed by mass spectrometry, and double-labelled experiments were carried out in order to detect and measure the incorporation of solvent moieties into M and R_M 4.33.

Figure 1 depicts the transformation of M into the single less polar form obtained in anhydrous neutral methanol (R_M 4.33) at 31°C. The reciprocal conversion of R_M 4.33 into M in water at the same temperature occurs at a higher rate than the former (Fig. 2). It is reasonable to believe that a prolonged exposure to methanol of the M form would curve the straight line of Fig. 1 into a similar hyperbola. Aqueous neutral methanol, containing up to 50% (by vol.) water, was equally efficient as anhydrous neutral methanol in converting M to R_M 4.33.

Anhydrous neutral ethanol and *n*-butanol, in which samples of M were stored up to 8 days, failed to transform M into less polar forms. In view of the protogenic nature of methanol *per se* and its possible influence on the reaction, we have studied the time course of these conversions in aqueous and alcoholic media to which acid had been added. Previously we determined the optimal concentrations of HCl for the

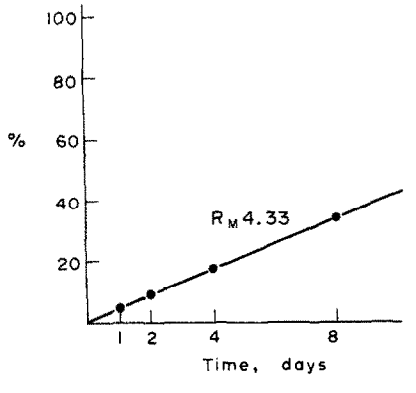

Fig. 1. Rate of conversion of M into R_M 4.33 in absolute methanol at 31°C.

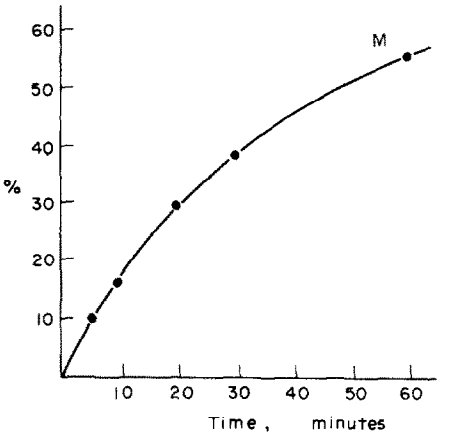

Fig. 2. Rate of conversion of R_M 4.33 into M in distilled water at 31°C.

formation of R_M^* 4.33 in water. This concentration was found to be equal to 10^{-2} N. At higher concentrations R_M^* 4.33 diminished noticeably, still less polar forms appearing in large amounts. Figure 3 shows the relative amount of the various compounds exhibiting different polarities, appearing at different time-intervals in acidic (10^{-2} N HCl) aqueous and acidic alcoholic (MeOH, EtOH, *n*-BuOH) media. It can be seen that the number of less polar forms is very similar in all these media, although R_M 1.84, one of the most abundant forms in alcoholic solutions, could not be detected in aqueous HCl. R_M 6.34 is also much more abundant in alcoholic acid media.

Mass spectra

Mass spectra were obtained for standard 18-OH-B, M, R_M 4.33 and R_M^* 4.33 at 150°C and 260°C, at 70 as well as 15 eV. Basically the same fragmentation patterns were obtained under the four conditions, except for differences in relative intensities.

M: M and standard 18-OH-B gave rise to identical patterns with a molecular ion at *m/e* 362 (Fig. 4).

R_M 4.33: the spectrum of R_M 4.33, possessing a molecular ion at m/e 376, is shown on Fig. 5. This spectrum could be assigned to the methyl-ketal represented on the same figure.

R_M^* 4.33: the relatively intense peak observed at *m/e* 344 can be plausibly ascribed to the molecular ion of a compound which would be formed from M by the loss of 18 mass units, *i.e.* a dehydration product (Fig. 6).

The probable structure of these compounds as well as the interpretation of other peaks will be discussed later.

Double-labelling experiments

[4-^{14}C]-Labelled R_M 4.33, prepared from [4-^{14}C]-18-OH-B as described in MATERIALS AND METHODS, was allowed to react with tritiated water. The resulting M was then isolated and the radioactive nucleide ratio was determined. The

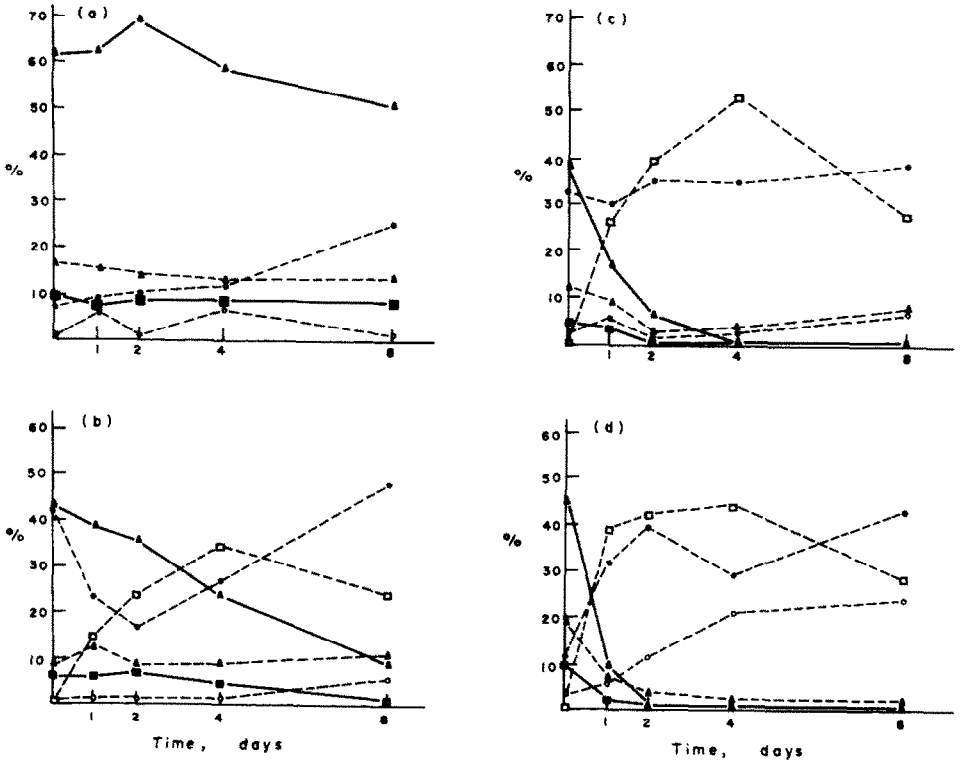

Fig. 3. Time course of the relative concentration of derivates in different acidic (10^{-2} N HCl) media: (a) water; (b) methanol; (c) ethanol; (d) *n*-butanol; all at 31°C. ■ M; □ R_M 1.84; ▲ R_M^* 4.33 for sub-figure (a) and R_M^{**} 4.33 for sub-figures (b), (c) and (d); △ R_M 5.26; ● R_M 6.34; ○ R_M 7.18. In all assays only pure M was used as starting material. The apparent presence of other derivates at "Zero time" ($\simeq$30 min) was due to the relatively fast formation of such derivatives.

product of the reciprocal conversion, R_M 4.33, produced by storage of this double-labelled M in radioinert neutral methanol, was also isolated and analysed. Table 1 shows the incorporation of water (tritium) when R_M 4.33 was converted into M and the loss of water when R_M 4.33 was regenerated (columns 2 and 4). The molar ratio of incorporated ^3H_2O to ^{14}C R_M 4.33 was equal to 0.75, while reformed R_M 4.33 only contained ^{14}C, thereby denoting that all the incorporated water was lost during the reverse reaction. The rather low value of tritium measured had to be expected because of the ready interchangeability with the medium of hydrogen atoms of the hemiketalic hydroxyl group at C-20

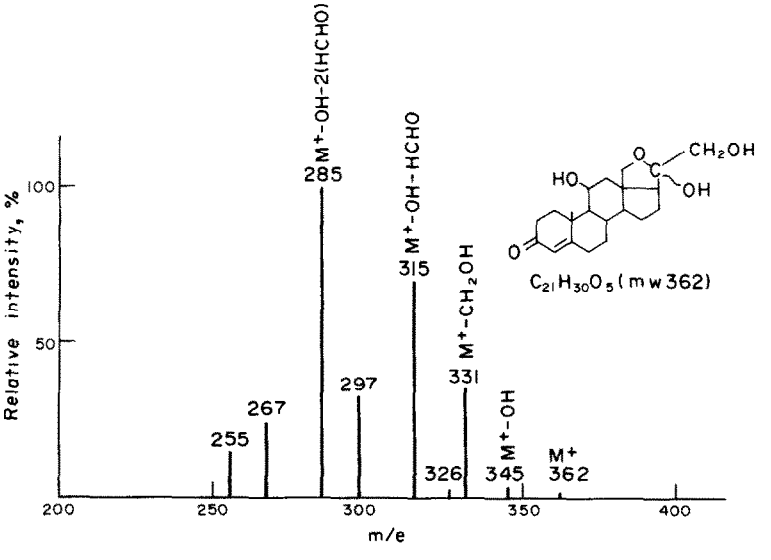

Fig. 4. Mass spectrum of 18-OH-B (M form).

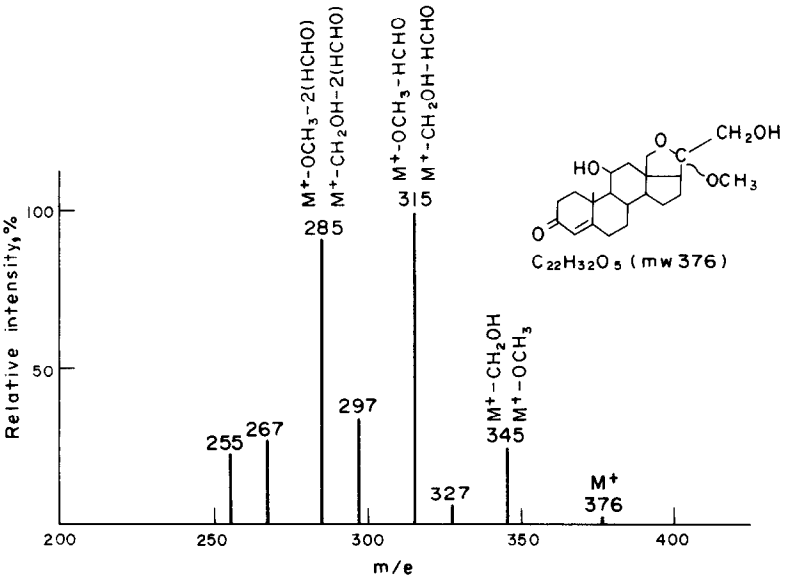

Fig. 5. Mass spectrum of R_M 4.33.

which resulted by hydrolysis of the proposed methyl-ketal structure shown in Fig. 5.

Conversely, [1, 2-^{3}H]-M was stored in anhydrous neutral $^{14}CH_3OH$ and the resulting R_M 4.33 was isolated and analysed. This conversion was also reversed upon dissolution and storage of the R_M 4.33 form, in radioinert water. The M thus obtained was isolated and its $^{14}C/^3H$ ratio determined. In this case, Table 1 shows the incorporation of $^{14}CH_3$ groups when M was transformed into R_M 4.33 and the loss of these methyl groups when M was regenerated (columns 1 and 3). It can be seen that the molar ratio of $^{14}CH_3OH$ incorporated into M was equal to 0.85; again, all the radioactivity due to incorporated methyl groups was lost when M was regenerated.

Stability and convertibility

M, R_M 4.33 and R_M^* 4.33, when stored *in vacuo*, in a dessicator, for over 20 days were found to remain as such.

R_M 4.33, but not R_M^* 4.33 could be reconverted to M upon dissolution in water.

DISCUSSION

These results indicate that 18 hydroxy-corticosterone is spontaneously converted into less polar forms upon storage in neutral methanol—anhydrous as well as aqueous—in methanol and higher aliphatic alcohols containing HCl and in aqueous HCl. Neutral higher alcohols failed to alter the more polar (M) form of the steroid. Figure 3 gives an idea of the time course of these spontaneous conversions. Table 2, in turn, summarizes the products formed as well as a tentative interpretation of their structures.

According to their mobilities in the Bush B_5 paper chromatographic system [11], at least five less polar

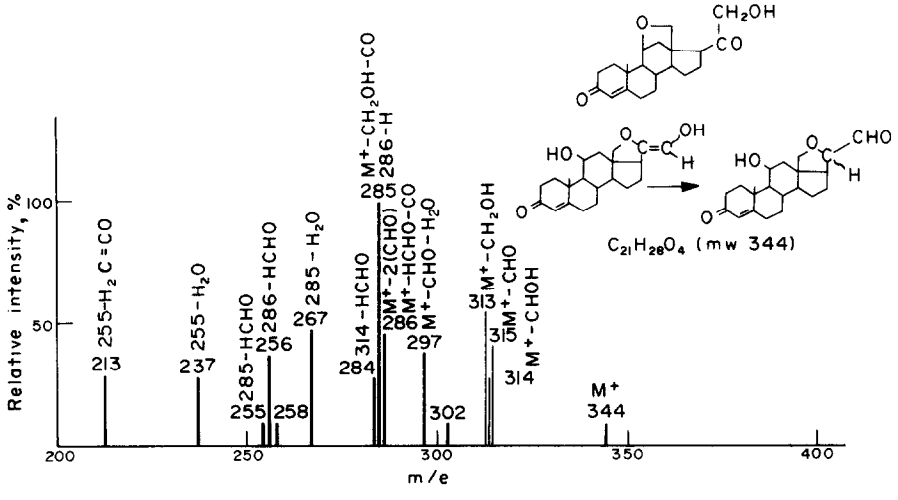

Fig. 6. Mass spectrum of R_M^* 4.33 (for details, see Discussion).

Table 1. Incorporation of $^{14}CH_3OH$ into [^{3}H]-M, and ^3H_2O into [^{14}C]-R_M 4.33 forms of 18-OH-B

Reactions	Molar ratios			
	Direct		Reversal	
	$^{14}CH_3OH$ incorporated / M	^3H_2O incorporated / R_M 4.33 (a)	$^{14}CH_3OH$ remaining / M reformed	^3H_2O remaining / R_M 4.33 reformed
[^{3}H]-M + $^{14}CH_3OH$ [^{14}C + ^{3}H]-R_M 4.33	0.85	—	0	—
[^{14}C]-R_M 4.33 + ^3H_2O [^{14}C + ^{3}H]-M	—	0.75	—	0

(a) Assuming 1 molecule of R_M 4.33 formed per molecule of M.

Reformed M was produced upon storage of [^{3}M + ^{14}C]-R_M 4.33 in water for 3 days at 31°C. Reformed R_M 4.33 was obtained from [^{3}H + ^{14}C]-M upon storage in radioinert neutral methanol for 10 days at 31°C.

Table 2. Treatment of 18-OH-B under various conditions: products formed and their interpretation

Treatment	Product	Interpretation
Neutral methanol	R_M 4.33	Methyl ketal at C-20
Aqueous HCl, 10^{-2} N	R^*_M 4.33	Dehydration products
Alcoholic HCl, 10^{-2} N	R^{**}_M 4.33	Structure not yet elucidated
Alcoholic HCl, 10^{-2} N	R_M 1.84	Structure not yet elucidated
Aqueous or Alcoholic HCl, 10^{-2} N	R_M 5.26	Structure not yet elucidated
Aqueous or Alcoholic HCl, 10^{-2} N	R_M 6.34	Structure not yet elucidated
Aqueous or Alcoholic HCl, 10^{-2} N	R_M 7.18	Structure not yet elucidated

compounds could be detected when the starting material —M—underwent some of the procedures described. A zone observed in all media is one possessing an R_M of 4.33. This zone, on the other hand, is the only less polar fraction appearing in neutral methanol (see Fig. 1). The zone appears immediately in all acidic media and remains unaltered when obtained in aqueous HCl (R^*_M 4.33 shown on Fig. 3). In acidic alcohols, the zone disappears with time at a rate increasing with the length of the aliphatic chain of the alcohols (R^{**}_M shown on Fig. 3). It is interesting to remark that R_M 4.33 originating from anhydrous neutral methanol is not identical to R^*_M 4.33 formed in aqueous HCl. Mass spectra of material from both sources were obtained and compared with the spectrum of the M form:

M yields a molecular ion at *m/e* 362 and a major peak at *m/e* 345 that can be ascribed to the loss of a hydroxyl group linked to C-11 or, more likely to C-20 of the hemiketalic structure. Standard 18-OH-B produces an identical mass spectrum (see Fig. 4).

R_M 4.33 shows a peak at *m/e* 376 that might be a molecular ion corresponding to a methyl-ketal at C-20. The loss of 31 mass units which accounts for the peak at *m/e* 345 can be ascribed to either the loss of the $-CH_2OH$ group or the $-OCH_3$ group, both at C-20. A further loss of a neutral fragment of formaldehyde, previously linked to C-20, could account for the loss of 30 additional units thereby producing the base peak at *m/e* 315. This fragment, in turn, by loss of a second formaldehyde molecule from C-18, could produce the important peak at *m/e* 285 (see Fig. 5). An analogous fragmentation would account for the peaks *m/e* 315 and 285 observed in the spectrum of M.

R^*_M 4.33 shows a peak at *m/e* 344 that could well be ascribed to a molecular ion corresponding to a dehydration product of the original M. The loss of water could occur between the hydroxyl groups at C-18 and C-11 with formation of an ether linkage. Alternatively, the water molecule could be derived from the hydroxyl group at C-20 and a hydrogen from C-21 of the hemiketalic structure; on further rearangement, a C-21 aldehyde could be formed (Fig. 6). The loss of 29 mass units, ascribed to a CHO fragment from this C-21 aldehyde, may produce the peak at *m/e* 315, an oxonium ion. This ion, by the loss of 18 mass units (water), could produce the peak at *m/e* 297 or, alternatively, by loss of 29 mass units corresponding to CHO from C-20, would give rise to the peak at *m/e* 286. This, in turn, would originate the base peak at *m/e* 285 by the loss of an H atom, producing a carbonium ion with an ether linkage between C-11 and C-18. Should the structure of R^*_M 4.33 be that of a C11-C18 ether previously mentioned, the loss of 31 mass units, CH_2OH from C-21, would account for the peak at *m/e* 313; a further loss of a neutral fragment of carbon monoxide would then allow an easy interpretation of the base peak at *m/e* 285, having the same structure as the base peak formerly rationalized.

The last fragmentation pattern described would indicate the absence of a previously existing hemiketalic system in R^*_M 4.33. This ion, *m/e* 285, by loss of a

formaldehyde molecule from C-18, would produce the peak *m/e* 255, which, losing a neutral fragment of ketene from ring A, 42 mass units, could produce the peak at *m/e* 213. Alternatively, losing 18 mass units, water, also from ring A, would give rise to the peak at *m/e* 237.

These results and their interpretation have to be analysed in conjunction with previous findings from this laboratory [5, 12] and others taken from the literature [1, 4, 6, 9]. Many of these studies were performed, not on 18-OH-B, but on the closely related homologue 18-hydroxy-11-desoxycorticosterone.

All but two [9, 12] previous publications, however, refer to a single less polar (L) zone whose structure has been the subject of various hypotheses. On the basis of preliminary investigations, methylketal [2, 8, 9] and condensed dimeric [2, 3, 5, 9, 13] structures have been proposed. Both of these could be probably ascribed to the lability of the hydrogen of the hydroxyl group linked to C-20 in the C18-C20 hemiketalic form. In addition to these two structures other workers [4, 13–16] have reported the formation of an 11β, 18 ether linkage in aqueous acid media.

The present work demonstrates the unsuspected complexity of the problem concerning less polar structures of, at least, 18-OH B: one single such fraction is found for 18-OH B stored in neutral methanol, but five fractions are obtained in acidic alcohols and four, in acidic aqueous solution. Each of these fractions, in turn, may or may not correspond to one single compound. At least two less polar zones with identical mobilities (R_M 4.33 and R_M^* 4.33) showed to possess different structures that most likely could be assigned, respectively, to a methylketal and dehydration products. Nothing is known about the structure of five zones with different mobilities that appear in acidic alcohols, including, again, one with an R_M of 4.33 ("R_M^{**} 4.33"). Nor have we any information about the structure of the three remaining zones appearing in aqueous HCl. Moreover, the presence of a mixture in R_M 4.33 cannot be excluded in view of a molecular ratio lower than unity in column 1 of Table 1 ($^{14}CH_3OH$ incorporated into [^{3}H]-M to form ($^{14}C^{+3}H)R_M$ 4.33). Whether this rather low value is due to a methodological or instrumental artifact or to a mixture of compounds remains to be elucidated. Works are in progress in our laboratory to achieve this purpose employing more precise techniques. These investigations also comprise structure elucidations and structure–property correlations of the above mentioned, as yet unknown fractions.

An intriguing biological problem arises from the fact that two different R_M 4.33 zones have been found to be relatively efficient precursors for aldosterone [5, 12]. In the second of these publications, care has been taken to individualize the particular L form, which proved to be R_M^* 4.33. This form could be spontaneously converted into the mineralocorticoid [12]. The 11β, 18 ether structure proposed above for this compound is closely related to the form of aldosterone which is expected to prevail in aqueous solution, especially at the temperature of homeothermic animals [9].

The elucidation and properties of this and all less polar structures might thus be of biochemical importance. Studies aimed at solving these problems are at present undertaken in our laboratory.

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