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EIGHTEEN-DEOXYALDOSTERONE AND OTHER LESS POLAR FORMS OF 18-HYDROXYCORTICOSTERONE AS ALDOSTERONE PRECURSORS IN RAT ADRENALS

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Summary—Samples containing as precursors either 18-hydroxycorticosterone (18-OH-B) in its M form, or this converted to less polar forms at pH 2 (ACM), or M or ACM enclosed in liposomes from adrenal lipids were incubated at pH 7.4, 4.8 or 3.3 in the presence or absence of quartered rat adrenals for 1 and 2 h. Optimal (10%) yields of aldosterone were obtained when (a) ACM was incubated at pH 4.8 and (b) M enclosed in liposomes was suspended in buffer and shaken without enzyme at pH 3.3. When conditions (a) were supplemented with malate and NADP, 16% of ACM was converted to aldosterone. ACM contained 80% of a fraction which, according to ^{13}C NMR spectroscopy, was identical to 18-deoxyaldosterone (18-DAL). Experiments in which radioactivity from corticosterone (B) or M was trapped by radioinert M or 18-DAL disclosed a pathway comprising sequentially B, 18-OH-B, 18-DAL and aldosterone, and the combined evidence of this work, an enzymatic hydroxylation of 18-DAL to aldosterone.

INTRODUCTION

It is well known that the usually isolated form of 18-OH-B[a], its more polar or "M" form [1, 2, 3] is a very poor precursor of aldosterone [4, 5, 6]. Investigations from this and other laboratories, however, suggest that certain less polar or "L" forms and derivatives of this hydroxylated steroid could be substrates for the mineralcorticoid [7, 8, 9].

Thus, according to preliminary studies, two of these less polar compounds migrating approx 4 times faster than 18-OH-B in the Bush B_5 chromatographic system [10] exhibited such precursor properties [7, 8].

One of these compounds, obtained by storage of 18-OH-B in methanol and later designated as R_M 4.33, is an acetal formed upon replacement of the enolic H of the 18-hydroxylated steroid by a methyl [2]. The other compound with the same mobility, designated R^*_M 4.33, was obtained by suspending 18-OH-B in aqueous HCl and exhibited, according to mass spectrometry, the structure of an anhydro-compound; this structure, however, permits two alternatives [2].

These preliminary results prompted the first part of the current work, a systematic investigation of conditions under which R^*_M 4.33 and a derivative similar to R_M 4.33 could be converted to aldosterone with relatively good yields, the latter derivative having been obtained by interaction of 18-OH-B with adrenal lipids instead of an artificial alcoxy donor such as methanol. Because acid pH influenced the formation and/or stability of both L forms [2, 7, 8], these studies were carried out in neutral as well as acid media.

Since in the course of the first part of this research 18-OH-B suspended in aqueous HCl showed to be a good precursor at pH 4.8, it was thought of interest to definitely characterize R^*_M 4.33, the main component of the resulting mixture. Employing ^{13}C NMR spectroscopy, which allowed to discriminate between the two possible anhydro-structures assigned to the fraction by mass spectrometry (see above), R^*_M 4.33 was finally identified as 18-deoxyaldosterone.

Once this identification had been achieved, 18-deoxyaldosterone itself was assayed as an aldosterone precursor and as an intermediate, a last precursor, on the pathway leading from corticosterone to aldosterone at pH 4.8. Concomitantly an experiment was designed aimed at confirming the

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[2]18-OH-B and 18-hydroxycorticosterone are synonyms of 11β,18,21-trihydroxy-4-pregnene-3,20-dione. B and corticosterone are synonyms of 11β,21-dihydroxy-4-pregnene-3,20-dione. Aldo and aldosterone are synonyms of 11β,21-dihydroxy-4-pregnene-3,18,20-trione mainly present under its 11β-18 hemiketalic and 11β-18, 18-20 bisketalic forms. 18-DAL and 18-deoxyaldosterone are synonyms of 21-hydroxy-11β,18-oxido-4-pregnene-3,20-dione. PPO is an abbreviation of 2,5-diphenyloxazole. Dimethyl POPOP is an abbreviation of dimethyl-1,4-*bis*(2-(5-phenyloxazole))-benzene.

hydroxylative nature of the conversion of 18-DAL to aldosterone at this pH.

EXPERIMENTAL

Precursor experiments

Forty-eight triplicate experiments consisting of the incubation of 18-OH-B in the presence or absence of quartered rat adrenals were carried out. Unless otherwise indicated, the conditions were those of Damasco and Lantos[7] and Aragonés *et al.*[8] the latter specially for transformations in the absence of tissue. A general outline of the experiments can be seen in Table 1. In these experiments, the conversion of the different forms of 18-OH-B to aldosterone was determined under combinations of conditions differing from each other by either: (a) the nature of the precursor (18-OH-B "M", or M previously suspended in 0.01 N HCl, the latter denominated "ACM" or "ACID M"); or (b) the enclosure, or lack of enclosure, of the precursor in liposomes; or (c) the pH of incubation media; or (d) time of incubation; or (e) the presence or absence of adrenal tissue.

(a) *Nature of the precursor. Precursor M*: To 0.7 μCi 18-OH-B were added 0.3 ml incubation medium; the mixture was shaken in a metabolic incubator at 37°C, either for 10 min (without liposomes or "nude") or 30 min (enclosed in liposomes); the precursor preparations thus obtained were then added to the remaining medium. *Precursor ACM:* To 0.7 μCi 18-OH-B were added 0.3 ml 0.01N HCl. The suspension was maintained under this condition over 1 min, the resulting precursor mixture containing 80% of a compound which was identified as 18-deoxyaldosterone (see Results). To this mixture were added 0.3 ml incubation medium. Finally the sample was shaken under conditions described for precursor M.

(b) *Liposomes containing either form of 18-OH-B.* These were obtained according to Abramson *et al.* [11] and Saunders *et al.* [12]. Briefly, an homogenate of 12 adrenals, or 240 mg "blank tissue" in 20% aqueous methanol was extracted twice with methylene chloride, the solvent was evaporated under nitrogen, the residue suspended in 3 ml methanol, stored overnight at 4°C and decanted. Total lipids were redissolved in 1 ml methylene chloride and 1 ml buffer and the organic solvent evaporated. Liposomes were then formed by sonicating the suspension during 20 s in a Cell Disrupter (Model 185 D, Heat System Ultrasonic Inc., Rathway NJ) at position 6 (130 watts/cm^2). Two ml of this emulsion were then added to 0.3 ml of suspension containing 0.7 μCi 18-OH-B, either in buffer or 0.01 N HCl. The formation of liposomes was checked under the microscope and the incorporation of the precursor, by scintillation-counting. Total lipids employed in the formation of liposomes were either obtained from adrenals or from a subcutaneous adipose pad ("liposomes from blank tissue").

(c) *Buffers*—pH 7.4: Krebs Ringer-bicarbonate-glucose, as prepared in [7].

pH 4.8: 0.001 M acetic acid-acetate (instead of phosphates), plus the remaining salts as in a). For experiments with malate and $NADP^+$ as cofactors, the buffer employed consisted of Tris–HCl plus the remaining salts as in a).

pH 3.3: 0.01 M PO_4H_3/PO_4H_2, plus the remaining salts as in (a). A final common ionic strength of 0.57 mol$^{1/2}$ was common to all media.

Separation methods and determination of aldosterone and its less polar derivative HLA

Aldosterone was separated, purified and quantified according to Kliman and Peterson[13]. HLA, the less polar compound recently found by Cozza *et al.*[14], was separated as indicated by these authors and quantified by isotope dilution.

Other precursor experiments

Twenty pmoles tritiated ACM were incubated at pH 4.8 at different time intervals. In another experiment, this radioactive precursor was incubated for 1.5 h in the presence of homogenate from 100 mg adrenal tissue, Tris–HCl buffer plus increasing amounts of sodium malate and 0.5 mM $NADP^+$.

Trapping experiments

(a) *Trapping of M from corticosterone.* 100 ± 5 mg quartered rat adrenals were incubated for 1.5 h with 25 pmol [1,2 ^{3}H]corticosterone and increasing quantities of radioinert 18-OH-B in its M form. Aldosterone was determined as above.

(b) *Trapping of 18-deoxyaldosterone from corticosterone.* As in (a), employing increasing amounts of 18-deoxyaldosterone instead of M.

(c) *Trapping of 18-deoxyaldosterone from M.* 25 pmol [1,2 ^{3}H]M were incubated as above in the presence of increasing amounts of 18-deoxyaldosterone.

Animals and materials

Rats. Male CHBB-Thom rats (200–250 g) from the animal house at the FCEN.

Radioactive steroids. 18-Hydroxy[1,2-^{3}H]corticosterone, 52 Ci/mmol, from The Radiochemical Center, Amersham, England. Aldosterone D-[4 ^{14}C], 57 Ci/mmol; and corticosterone [1,2-^{3}H], 40 Ci/mmol, from New England Nuclear, Cambridge, MA, U.S.A.

Radioinert steroids. 18-Hydroxycorticosterone, from Makor Chemicals, Jerusalem, Israel. 18-Deoxyaldosterone 21-acetate was synthesized as in [15]. 18-Deoxyaldosterone was prepared by hydrolysis of the former as follows: 18 mg of the acetate were dissolved in 0.26 ml methylene chloride plus 0.83 ml methanol; concentrated HCl (0.12 ml) and water (0.06 ml) were added and the mixture was vigorously stirred for 44 h and then diluted with methylene chloride. The organic layer was separated, washed successively with $NaHCO_3$ solution and water, dried

with $MgSO_4$ and evaporated, yielding 18-deoxyaldosterone homogenous by TLC.

When 18-deoxyaldosterone was prepared from the M form of 18-OH-B, 1.1 mg of this steroid was dissolved in Cl_3CD (0.35 ml) and gaseous HCl was bubbled through the solution for a few seconds. The solution was used directly for NMR analysis. Evaporation to dryness rendered 18-deoxyaldosterone, identical to that obtained by hydrolysis of its acetate.

Drugs and cofactors. All inorganic salts and sodium malate were of reagent grade. $NADP^+$ was from Sigma Chemicals.

NMR spectra

^1H NMR spectra were determined at 300 MHz in a Bruker WB-300. ^{13}C NMR spectra were measured at 75 MHz in a Bruker WB-300 or at 25.2 MHz in a Varian XL-100-15 operating in the FT-mode with broad band ^1H decoupling.

Scintillation counting and radioactivity scanning

A Packard radioactivity scanner, Model 7201 was used to detect radioactive zones on paper chromatograms and TLC plates. A Tracor Analytic Liquid Scintillation System, Model 6882 was employed for counting the radioactivity in samples containing scintillation cocktail (3 g PPO and 300 mg dimethyl POPOP per 1 toluene).

Statistical methods

Factorial analyses of variance were processed with a VAX 11/750 computer (Instituto de Cálculo de la Facultad de Ciencias Exactas y Naturales).

RESULTS

Expression of the yields of aldosterone

In these results aldosterone is expressed as the sum of the usually isolated form, plus a less polar derivative, HLA, formed spontaneously and reversibly upon storage of the steroid in acid medium or, as a minor metabolite, in incubations. The proportion of HLA in total aldosterone varied between 5 and 10%. The properties and characterization of HLA will be the subject of a forthcoming publication [14].

Optimal conditions for aldosterone formation

An analysis of Table 1 excludes 7.4 as suitable pH for the formation of aldosterone. At this pH aldosterone yields are almost constantly below those corresponding to one or both lower pH levels (horizontal lines A to F and I to P). Only two lines, G and H, exhibit slightly higher yields at pH 7.4, but these yields are far from being optimal.

For those more acid pH levels, a 4-factor analysis of variance, followed by contrasts, reveals that two conditions are different from all others, that they are optimal and that they do not differ quantitatively from each other: incubations of nude ACM, at pH 4.8 (pH 4.8—line D), and spontaneous conversions of M, enclosed in adrenal liposomes, at pH 3.3 (pH 3.3—line N). Not listed in the table are conversions obtained with precursors enclosed in liposomes from a subcutaneous pad: they are insignificant. A third condition, incubation of nude M at pH 3.3 (pH 3.3—line B) exhibits an equally high mean value but its variance, due to highly disperse triplicate values, comprises an interval containing all other intervals. (For more statistical details, see legend to Table 1).

Of these conditions, "pH 4.8—line D" are the most interesting ones because some organelles in the living cell possess approximately this pH (see Discussion) and because under these conditions, in contrast to the other two optimal conditions mentioned, relatively high conversion values may be ascribed mainly to an enzymatic reaction. (Table 1: deduct line H from line D in pH 4.8 column).

The characterization of the main component of ACM and a series of experiments designed to confirm the role of this component, which was identified as 18-deoxyaldosterone, as an intermediate in the overall conversion of corticosterone to aldosterone, were therefore undertaken.

The nature of ACM and of R^*_M 4.33

ACM, as prepared in this work, is a mixture of four radioactive fractions, 80% of which correspond to R^*_M 4.33 of Aragonés *et al.*[2]. When the medium is further acidified by bubbling gaseous HCl, 98% of the mixture is transformed into R^*_M 4.33. The structure of R^*_M 4.33, was determined from ^{13}C and ^1H-NMR data and confirmed to be identical to 18-deoxyaldosterone by comparison with this compound obtained by acid hydrolysis of 18-deoxyaldosterone-21-acetate [15]. The ^1H and ^{13}C-NMR data of the compounds studied are summarized in Tables 2 and 3. R^*_M 4.33 was prepared *in situ* by bubbling HCl vapors for a few seconds through a solution of 18-OH-B in deuterochloroform, and the resulting solution was used directly for NMR analysis (the use of deuterium chloride vapors resulted only in a lower conversion yield). The presence of low intensity resonance lines apart from those listed in Table 3 indicated the occurrence of one or more minor compounds in ACM apart from the main component R^*_M 4.33.

It was concluded that ACM contained approx 80% R^*_M 4.33 and that this radioactive fraction is identical to 18-deoxyaldosterone.

Time course of formation of 18-deoxyaldosterone and aldosterone at pH 4.8

Table 4 contains the time courses of the biosyntheses of 18-deoxyaldosterone and aldosterone when M was used as a precursor. It can be seen that 18-deoxyaldosterone behaves like an intermediate, and aldosterone, like an end product of the reaction. Not listed in the table is a fraction R_M 6.33, which

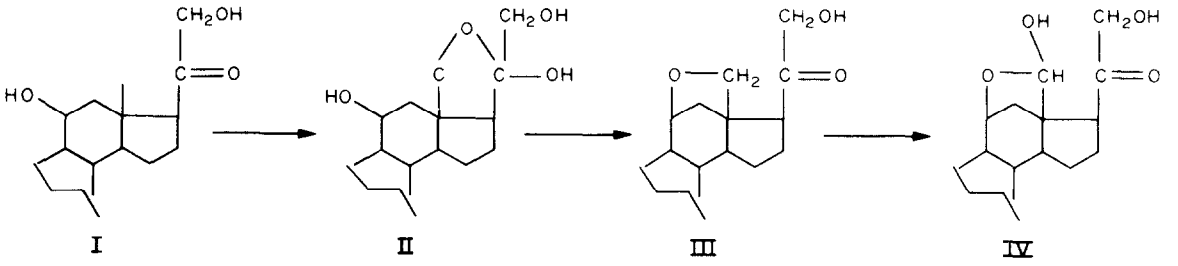

Fig. 1. Compounds tested as intermediates along the pathway from corticosterone to aldosterone. I, corticosterone; II, 18-OH-B (M form); III, 18-deoxyaldosterone; IV, aldosterone.

also possesses the kinetic characteristics of an end product.

Trapping experiments at pH 4.8

The series of preceeding experiments favors the concept of a pathway M→18-deoxyaldosterone→aldosterone. That 18-OH-B, even at pH 7, is an intermediate, however poor, between corticosterone and aldosterone, has already been shown by means of trapping assays by Vecsei *et al.* [16] and Kojima *et al.* [17]. Similar experiments were therefore performed at pH 4.8, in which radioactivity from precursors along the overall postulated pathway was trapped by presumed intermediates. The pathway can be visualised in Fig. 1. Results of Table 5 and 6 indicate that M, as well as 18-deoxyaldosterone, are its echelons. A variant of this interpretation is discussed at the end of the paper.

DISCUSSION

A systematic investigation of conditions under which 18-OH-B possessed precursor properties disclosed two optimal combinations for its conversion to aldosterone: (a) The steroid enclosed in adrenal liposomes, in the absence of enzyme, at pH 3.3; and (b) its acid or ACM form, in the presence of adrenal tissue, at pH 4.8. For several reasons, combination (b) was thought to be of special interest. Four point eight is, indeed, a pH compatible with certain organelles in the living cell, such as lysosomes [18], receptosomes [19] and, according to Mitchell's chemiosmotic theory [20], the outer phase of the inner membrane of mitochondria. Furthermore, under these combined conditions at variance with (a), relatively high conversion values could be ascribed to an enzymatic reaction. (Deduct line H from line D in the pH 4.8 column of Table 1).

Since ACM was a good precursor for aldosterone, it became important to identify its main component. In a previous work, this component had been tentatively characterized by mass spectrometry as an anhydro compound to which two alternative structures could be assigned [2]. In the current investigation, ^{1}H and ^{13}C-NMR spectral data, com-

Table 1. Precursor conversions to aldosterone

Enclosure	Type of conversion	Precursor	Time (hours)	pH 3.3	pH 4.8	pH 7.4	Horizontal line
Nude	Enzymic	M	1	6.93 ± 2.21	6.64 ± 0.76	0.67 ± 0.02	A
			2	9.45 ± 2.37	7.12 ± 0.33	0.73 ± 0.01	B
		ACM	1	4.92 ± 1.43	8.66 ± 0.71	4.26 ± 0.02	C
			2	5.88 ± 1.52	9.66 ± 0.53	5.57 ± 0.06	D
	Spontaneous	M	1	3.60 ± 0.50	1.03 ± 0.15	1.00 ± 0.02	E
			2	5.04 ± 1.71	1.32 ± 0.05	1.18 ± 0.03	F
		ACM	1	3.65 ± 0.58	2.65 ± 0.13	4.37 ± 0.02	G
			2	5.21 ± 1.07	3.13 ± 0.12	5.70 ± 0.04	H
Liposomes	Enzymic	M	1	1.72 ± 0.06	3.88 ± 0.27	2.66 ± 0.19	I
			2	1.89 ± 0.22	4.42 ± 0.36	3.81 ± 0.11	J
		ACM	1	1.68 ± 0.23	5.68 ± 0.45	2.01 ± 0.04	K
			2	2.88 ± 0.27	7.02 ± 0.97	2.89 ± 0.13	L
	Spontaneous	M	1	9.90 ± 0.67	7.32 ± 1.10	1.62 ± 0.02	M
			2	10.45 ± 1.43	7.42 ± 0.92	1.68 ± 0.11	N
		ACM	1	6.78 ± 0.68	5.41 ± 0.50	3.61 ± 0.37	O
			2	8.12 ± 1.02	6.33 ± 0.60	5.47 ± 0.08	P

The table lists combinations of conditions as described in Experimental. Values are means of triplicates ± Standard Errors. For statistical purposes, conditions are categorized as "factors" which comprise: enclosure (nude or liposomes), type of conversion (enzymic or spontaneous), precursor (M or ACM), time (1 h or 2 h) and pH (3.3, 4.8 or 7.4). A 5-factor ANOVA discloses differences ($P < 0.01$) within factor pH ($\nu_1 = 2$, $\nu_2 = 96$). These differences disappear upon elimination of pH level 7.4. Factor time is significant for the remaining pH levels ($\nu_1 = 1$, $\nu_2 = 60$), 2 h being more efficient than 1 h. Fourth order interaction enclosure-conversion-precursor-pH (2 levels) is significant for time = 2 h ($\nu_1 = 1$, $\nu_2 = 60$). There is no significant difference between pH 4.8—line D and pH 3.3—line N which are both optimal; but nothing can be stated about pH 3.3—line B, since its variance comprises an interval containing all other intervals.

Table 2. ^{1}H NMR spectral data in deuterochloroform at 300 MHz

	18-OH-B	18-OH-B + HCl
H-4	5,70 (bs)	5,76 (bs)
H-11	4,43 (bt, J = 3 Hz)	4,43 (bd, J = 6 Hz)
H_A-18	4,27 (d, J = 10 Hz)	3,73 (d, J = 9 Hz)
H_B-18	3,80 (d, J = 10 Hz)	3,36 (d, J = 9 Hz)
H_A-21	3,82 (dd, J = 10 Hz, J = 12 Hz)	4,23 (s)
H_B-21	3,65 (dd, J = 10 Hz, J = 12 Hz)	
20-OH	3,32 (s)	
21-OH		3,02 (bs)
CH_3-19	1,41 (s)	1,28 (s)

Chemical shifts are in ppm downfield from tetramethylsilane. d: doublet; dd: double doublet; s: singlet; bs: broad singlet; bt: broad triplet; bd: broad doublet.

Table 5. Effect of increasing amounts of 18-OH-B "M" on the production of aldosterone from corticosterone at pH 4.8 (See "trapping experiments" in Experimental)

"M" (ng)	% Aldosterone-^{3}H
0	11.3
1	8.0
10^2	4.9
10^4	2.6
10^6	1.7

pared to those of a synthetic sample, lead to its definitive identification as 18-deoxyaldosterone. Ulick had already encountered a 3.8% conversion of this intramolecular ether to aldosterone by bullfrog adrenal slices [9]. The current findings show even higher conversions by rat adrenals at pH 4.8 and demonstrate that 18-DAL is under these conditions an intermediate on the pathway leading from the M form of 18-OH-B to aldosterone. In effect, 18-DAL behaves as an intermediate, and aldosterone behaves as an end product, when M is used as a precursor at pH 4.8 (Table 4). Furthermore, the yield of [^{3}H]aldosterone could be diminished upon addition of radioinert 18-DAL in a dose-dependent manner when either [^{3}H]corticosterone or the M form of [^{3}H]18-OH-B were used as precursors (Table 6). These latter results strongly suggest that [^{3}H]18-DAL derived from each of these precursors was diluted with the exogenously added 18-DAL thereby causing a reduction of the yields of [^{3}H]aldosterone.

But the final and most important proof for the precursor properties of 18-deoxyaldosterone consisted of the yields of aldosterone obtained when incubation samples containing the intramolecular ether were supplemented with an NADPH generating system (Table 7). Conversions under these conditions

Table 3. ^{13}C-NMR spectral data in deuterochloroform

Carbon	18-deoxyaldosterone[a] -21-acetate	18-deoxyaldosterone[a]	18-HO-B[b]	18-HO-B + HCl[b]
1	36.73	36.74	35.47	36.81
2	36.00	36.01	32.13	36.08
3	198.92	199.05	199.20	201.09[c]
4	124.14	124.18	122.59	124.38
5	169.85	169.83	171.80	170.20[c]
6	33.61	33.62	33.83	33.64
7	32.76	32.75	32.94	32.84
8	31.37	31.35	32.01	31.45
9	50.59	50.49	(55.04)	50.60
10	38.56	38.57	35.17	38.68
11	74.42	74.51	65.77	74.63
12	42.73	42.87	46.48	42.98
13	53.77	53.89	52.48	54.60
14	54.32	54.28	(56.36)	54.40
15	(25.51)	(25.58)	[25.25]	25.63
16	(25.98)	(26.05)	[26.01]	26.10
17	58.23	58.24	(56.75)	58.38
18	68.91	69.04	73.41	69.12
19	18.63	18.65	21.36	18.73
20	203.25	209.51	105.95	210.00[c]
21	68.91	69.49	68.60	69.58
$\underline{CH_3}CO$—	20.36	—	—	—
$\underline{CH_3}CO$—	169.85	—	—	—

[a] ^{13}C-NMR at 25.2 MHz.
[b] ^{13}C-NMR at 75 MHz.
[c] Small differences observed with respect to 18-deoxyaldosterone spectrum may be ascribed to the presence of dissolved HCl.
Values in parentheses or in square brackets may be interchanged. Chemical shifts are in ppm downfield from tetramethylsilane.

Table 4. Time course of the formation of 18-deoxyaldosterone and aldosterone from M at pH 4.8

Incubation time (h)	Remaining M-^{3}H (dpm × 10^{-4})	18-Deoxyaldosterone-^{3}H (dpm × 10^{-4})	Aldosterone-^{3}H (dpm × 10^{-4})
0.5	110.08	3.72	1.95
1.0	93.02	4.63	6.02
1.5	82.56	8.50	7.78
2.0	68.20	5.41	9.04
2.5	56.91	3.39	10.02

Table 6. Effect of increasing amounts of 18-deoxyaldosterone on the production of aldosterone from corticosterone and 18-OH-B ("M" form) at pH 4.8

18-Deoxyaldosterone (ng)	% Aldosterone-^{3}H from B-^{3}H	% Aldosterone from M-^{3}H
0	10.5	7.0
1	7.1	6.2
10^2	3.7	4.3
10^4	1.3	2.1
10^6	0.5	0.5

amounted to 16%. These conversions, the highest so far obtained for any 18-OH-B form, are 4–8 times greater than those achieved with rat adrenal preparations at a neutral pH when the M form was used as a substrate, and are at least 50% higher than conversions from corticosterone by these preparations (compare to [7], [17], [23], [27] and [28] amongst others). In agreement with early ideas of Ulick [21] and Finkelstein and Schaefer [22] and in line with the findings of Marusic *et al.*[23], Kojima *et al.*[17] and Aupetit *et al.*[24] this experiment demonstrated concomitantly that the enzymatic transformation of 18-DAL to aldosterone is an hydroxylation. The fact that the spontaneous transformation of the M form of 18-OH-B to 18-deoxyaldosterone needs, and takes place rapidly in acid media, even at pH 4.8, favors the concept of an initial, spontaneous sub-step in the last, complex step of aldosterone biosynthesis. This transformation could well occur by one of two alternative dehydrations proposed in Fig. 2, both depending on the presence of a free proton. But the requirement of an acid medium also for the second sub-step, which according to evidence discussed above is an enzymatic one requiring reducing power and cytochrome P450, needs an additional explanation. If the reaction occured as shown in Fig. 3 then an excess of protons would be critical in order not to displace the non-enzymatic sub-step towards M (See Fig. 4). In other words, an acid medium, one proton, would be necessary to trigger the spontaneous conversion of 18-OH-B M to 18-deoxyaldosterone and a second

Table 7. Effect of cofactors and malate on the production of aldosterone from M and ACM at pH 4.8

Malate (mM)	NADP 0.5 mM	NADPH 0.5 mM	% Aldosterone-^{3}H from M-^{3}H	% Aldosterone-^{3}H from ACM-^{3}H
—	—	+	4.98	5.84
—	+	—	2.98	4.62
0.15	+	—	5.37	6.56
0.60	+	—	6.45	8.92
1.20	+	—	8.67	11.24
3.00	+	—	11.58	15.67
9.00	+	—	13.27	16.22
9.00			7.48	8.09
—	—	—	3.24	4.41

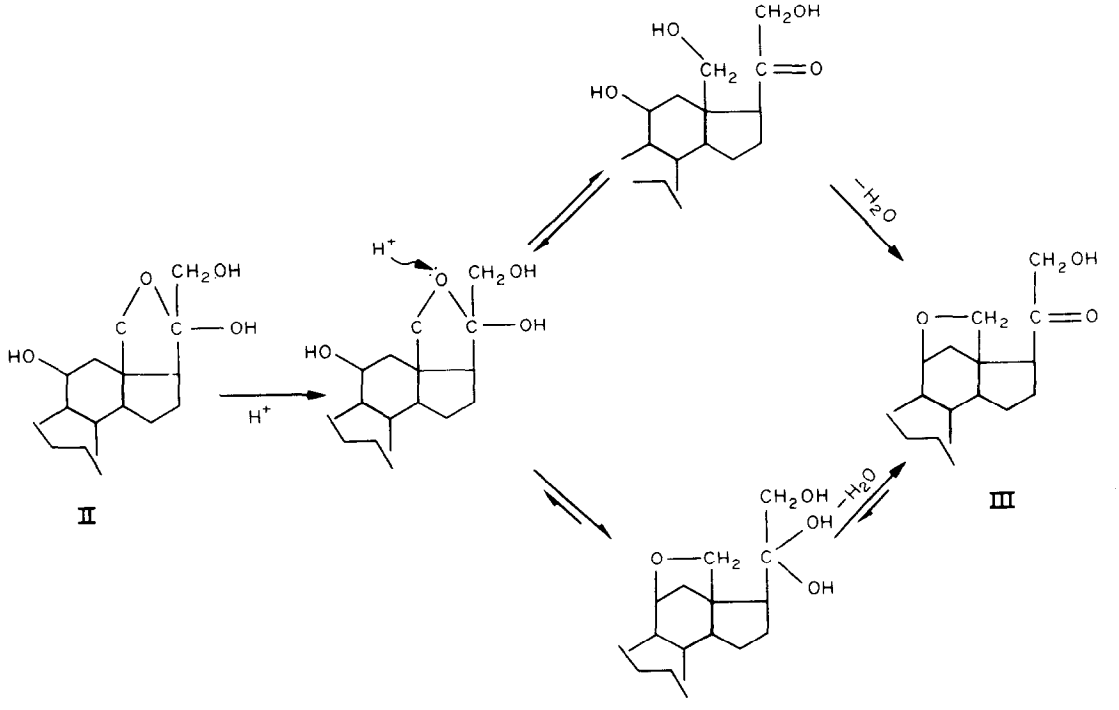

Fig. 2. Dehydration mechanisms postulated for the transformation of M to 18-deoxyaldosterone. For the significance of Roman numbers, see Fig. 1.

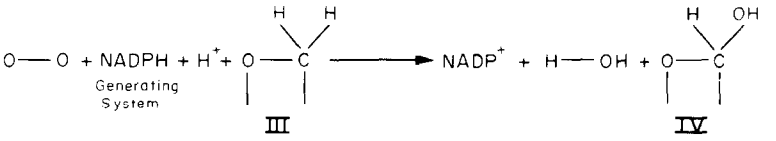

Fig. 3. Hydroxylation of 18-deoxyaldosterone. For the significance of Roman numbers, see Fig. 1.

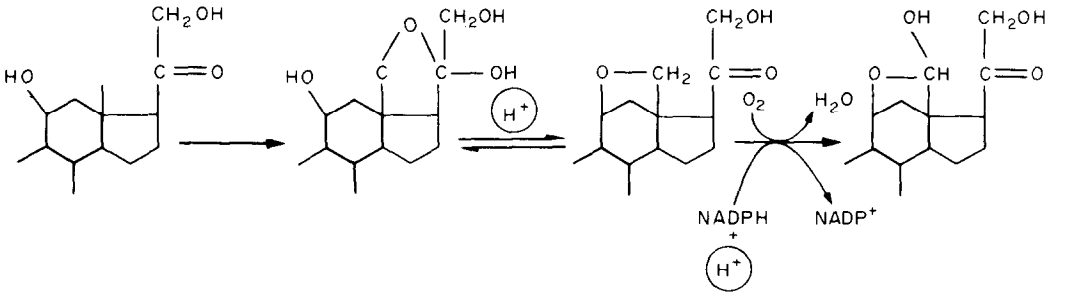

Fig. 4. Proposed aldosterone-pathway at pH 4.8.

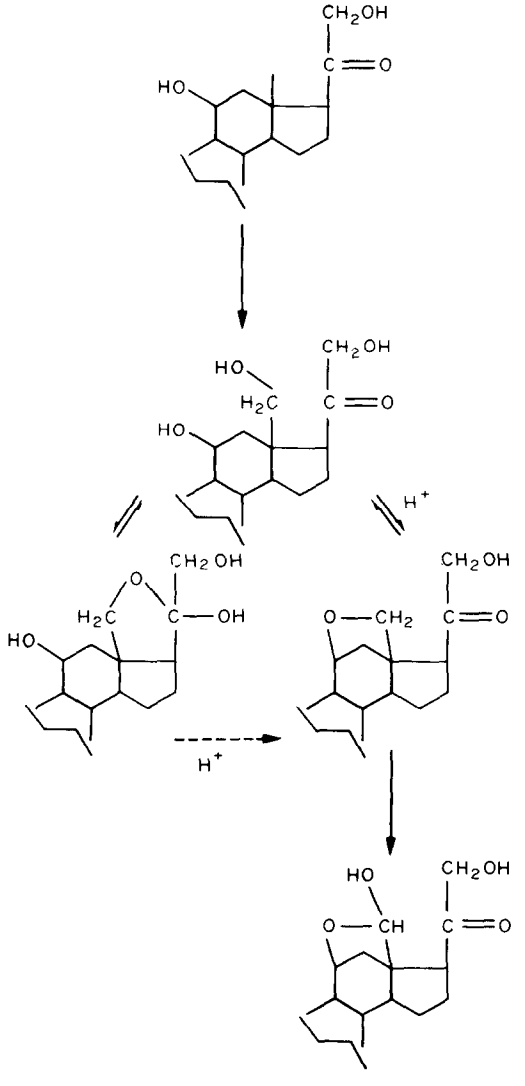

Fig. 5. Alternative aldosterone pathways at pH 4.8.

proton, to favor the enzymatic hydroxylation of this intermediate towards aldosterone.

The high yields obtained spontaneously at pH 3.3 with 18-OH-B enclosed in adrenal liposomes (pH 3.3—line N), and the more questionable ones, with incubations of M at this pH in the presence of adrenal tissue (pH 3.3—line B) are noteworthy. However, not only their biological meaning but also their mechanism remains obscure. One finding difficult to explain is that M should be all of a sudden a slightly better precursor than ACM at pH 3.3. It is possible that under conditions of pH 3.3—line N the M form of 18-OH-B could interact with lipids, probably membrane lipids of the adrenal cell. It is noteworthy in this respect that 18-OH-B M enclosed in liposomes from a subcutaneous adipose pad does not possess precursor properties. The interaction with adrenal liposomes could well be of the 20-alcoxy type found, amongst others, by Aragonés *et al.*[2] and by Usa *et al.*[25]. Both groups describe how this ketalization is catalysed by traces of acid. Why this spontaneous mechanism, however, leads to high yields of aldosterone is still unknown.

As early as 1964, Pasqualini[26] demonstrated that cortico-adrenal tissue, as well as adrenal tumor from a patient with Conn's syndrome, was capable of converting tritiated 18-OH-B to aldosterone. The author incubated homogenates of these tissues, supplemented with NAD and fumarate, at pH 7.4. Aldosterone yields of these incubations, although below those obtained in the current work at pH 4.8 with 18-deoxyaldosterone in the presence of NADP and malate, are nevertheless important and it would be of interest to study the effect of similar pathological preparations on the structure of 18-OH-B. The possibility exists, indeed, that these tissue samples could stabilise the 18-deoxyaldosterone form, or any other biosynthetically active L form of 18-hydroxycorticosterone.

These different routes to aldosterone, as well as pathways bypassing 18-OH-B altogether, such as those described in [27] and [28], reaffirm the complexity of ion-regulation through mineralocorticoids which, in view of its biological importance, cannot be confined to a single line of enzymatic mechanisms leading to the strongest sodium-retaining hormone. The pathways represented in Figs 4 and 5, the latter in better agreement with the mechanisms postulated in Fig. 2 and with the ideas of others [21], constitute two alternatives for this regulation ensuing from those experiments that have been carried out at pH 4.8.

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