Aldosterone Biosynthesis from 4-^{14}C-Pregnenolone and 4-^{14}C-Progesterone, in the Rat and Rabbit (37501)

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In vitro experiments using 4-^{14}C-pregnenolone and 4-^{14}C-progesterone were performed in order to study the pathways to aldosterone formation in adrenal glands from rats and rabbits. The adrenal steroids were isolated by paper chromatography and quantitated by the double isotope derivative assay method. In both the rat and the rabbit, there seemed to be no distinction between the roles of pregnenolone and progesterone as precursors of adrenal steroids. Corticosterone, 11-dehydrocorticosterone, and 18-hydroxycorticosterone were possible precursors of aldosterone in the rabbit, whereas in the rat the precursors were corticosterone, 18-hydroxycorticosterone, and 18-hydroxy-11-deoxycorticosterone. The immediate precursor of aldosterone in both species was 18-hydroxycorticosterone.

Introduction. The biosynthetic pathway to aldosterone[4] has aroused considerable discussion in the past ten years since it was found to be the main mineralocorticoid in several species including man. Pregnenolone, progesterone, 11-deoxycorticosterone (DOC), and corticosterone (B_K) serve as precursors for the biosynthesis of aldosterone (1–9). Of these steroids, B_K appears to be the more immediate precursor (2). The identification of 18-hydroxy-11-deoxycorticosterone (18-OH DOC) and 18-hydroxycorticosterone (18-OH B_K) in many different species provides the basis for an alternate pathway which remains controversial (10–14). Fazekas and co-workers (15–18) reported the conversion of 11-dehydrocorticosterone (A_K) to aldosterone via 18-hydroxy-11-dehydrocorticosterone (18-OH A_K) by rabbit adrenal tissue and by homogenates of adrenal adenomas and normal adjacent cortex of patients with Conn's syndrome.

Presently, there exists much conflicting data concerning the biosynthetic pathways to aldosterone in different species. This is particularly true in rats and rabbits. For this reason, a series of *in vitro* experiments using 4-^{14}C-pregnenolone and 4-^{14}C-progesterone as precursors were performed to determine as many intermediates as possible to aldosterone. This was done in order to elucidate the pathway to aldosterone. Basically, the results indicate that the immediate precursor of aldosterone in both the rat and the rabbit appears to be 18-OH B_K.

Methods. Experiment I. Twenty-four

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[4] The following trivial names and abbreviations are used in this paper: Pregnenolone (preg): pregn-5-en-3β-ol-20-one. Progesterone (prog): pregn-4-en-3,20-dione. 17α-hydroxypregnenolone (17α-OH-preg): pregn-5-en-3β, 17α-diol-20-one. 17α-hydroxyprogesterone (17α-OH prog): pregn-4-en-17α-ol-3,20-dione. 11-deoxycorticosterone (DOC): pregn-4-en-21-ol-3,20-dione. 18-hydroxy-11-deoxycorticosterone (18-OH DOC): pregn-4-en-18,21-diol-3,20-dione. Corticosterone (Kendall's compound B, B_K): pregn-4-en-11β,21-diol-3,20-dione. 18-hydroxycorticosterone (18-OH B_K): pregn-4-en-11β,18,21-triol-3,20-dione. 11-dehydrocorticosterone (Kendall's compound A, A_K): pregn-4-en-21-ol-3,11,20-trione. Aldosterone: pregn-4-en-11β,21-diol-18-al-3,20-dione. 11-deoxycortisol (Reichstein's compound S, S_R): pregn-4-en-17α,21-diol-3,20-dione. Cortisol (Kendall's compound F, F_K): pregn-4-en-11β,17α,21-triol-3,20-dione. Cortisone (Kendall's compound E, E_K): pregn-4-en-17α, 21-diol-3,11,20-trione. PPO-POPOP: 2,5-diphenyloxazole-1,4-di-2(5 phenyloxazole).

white male rabbits weighing between 2.3 and 4.6 kg were maintained on Purina rabbit pellets and had water *ad libitum*. The animals were sacrificed by a sharp blow on the back of the neck. The right and left adrenals were immediately removed, cleaned of fat, weighed, and cut into halves. One half of the right and left adrenals were weighed and then added to a flask containing 212 nmole of 4-^{14}C-pregnenolone with an initial specific activity of 912 cpm/nmole, whereas the other halves were weighed and added to a flask containing 208 nmole of 4-^{14}C-progesterone with an initial specific activity of 923 cpm/nmole. Each of the above flasks also contained 5 ml of Krebs–Ringer bicarbonate buffer with 250 mg% glucose and a drop of propylene glycol. The flasks were incubated in a Dubnoff metabolic shaker for 2 hr at 37° in an atmosphere of 95% O_2 and 5% CO_2. The contents of each flask were extracted three times with ethyl acetate and concentrated in a pointed tube. The steroids were isolated for quantitation by paper chromatography using different Zaffaroni systems (19). A radioactive strip counter was then used in locating the radioactive tracers added. Regions which ran with authentic standards of the steroids to be quantitated were eluted from the paper using spectroquality methanol and acetylated with acetic-^{3}H-anhydride for quantitation by the double isotope derivative assay method. After running the acetylated steroids in at least three different Zaffaroni systems, the regions which ran with the standards, the regions on both sides, and the distilled water blanks were eluted, and a toluene PPO-POPOP cocktail containing an aliquot was then counted in a Packard Tricarb Liquid Scintillation Spectrophotometer Model 3003 set for counting ^{3}H and ^{14}C simultaneously.

The specific activity of the acetic-^{3}H-anhydride was determined by a modification of the Kliman and Peterson method (20). Details of the isolation, characterization, quantitation, and criteria of purity of the isolated steroids have been reported (21, 22).

Experiment II. Twenty-four Sprague–Dawley male rats weighing between 180 and 450 g were sacrificed by decapitation, and the adrenals were immediately removed and treated in the same manner as in Expt I, except different quantities and specific activities of the precursors were used. The rat adrenals were incubated with 132 nmole of pregnenolone and 134 nmole of progesterone with specific activities of 1442 and 1424 cpm/nmole, respectively. As stated above the remainder of the procedure was the same as in the rabbit experiment.

Results. Experiment I: Effects of incubating adrenal glands from rabbits with 4-^{14}C-pregnenolone (S.A. = 912 cpm/nmole) or 4-^{14}C-progesterone (S.A. = 923 cpm/nmole). The rabbit adrenal steroid production in nanomoles per two hours of incubation and specific activities are illustrated in Table I. All steroid values have been corrected for losses that occurred during their isolation and characterization. This usually ranged between 40 and 60% except for progesterone which ranged between 70 and 90% due to the reduction step before acetylation. The combined adrenal gland weights were 331 ± 30.5 mg whereas the range of adrenal weights per incubation was 118–290 mg.

Only the pertinent steroids to aldosterone formation are depicted in Table I. Besides these steroids, 17α-hydroxy pregnenolone (17α-OH preg), 11-deoxycortisol (S_R), and cortisone (E_K) were quantitated and only a trace of 17α-hydroxy progesterone (17α-OH prog) was detected. 18-OH A_K was tentatively identified according to its polarity in different chromatographic systems. Table I illustrates that the total nanomolar quantities and specific activities of DOC from 4-^{14}C-pregnenolone or 4-^{14}C-progesterone were not significantly different. With one exception, this was the case for the other steroids listed in Table I.

In most cases there was a tendency for the quantities and specific activities of the above compounds to be higher from 4-^{14}C-progesterone than from 4-^{14}C-pregnenolone. However, if one considers that progesterone is one step further down the biosynthetic scheme than pregnenolone, this trend could be explained on this basis alone.

Nevertheless, it should be noted from Table I that the specific activities decrease from DOC to A_K in all cases. This seemed

TABLE I. Rabbit Adrenal Steroid Production (nmole/2 hr of incubation) and Steroid Specific Activity (S.A.) (cpm/nmole) Following Adrenal Gland Incubations with 4-^{14}C-pregnenolone (4-^{14}C-preg, S.A. = 912 cpm/nmole) or 4-^{14}C-progesterone (4-^{14}C-prog, S.A. = 923 cpm/nmole).[a]

	4-^{14}C-preg		4-^{14}C-prog	
	nanomoles	S.A.	nanomoles	S.A.
Preg	15.74 ± 2.72	694 ± 102	ND	ND
Prog	18.51 ± 4.92	647 ± 89.0	22.05 ± 2.83 n.s.[b,c]	732 ± 71.3 n.s.[b,c]
DOC	28.43 ± 6.36	650 ± 69.0	32.64 ± 6.8 n.s.[b,c]	710 ± 82.0 n.s.[b,c]
B_K	60.20 ± 7.37	603 ± 21.6	78.90 ± 5.8 n.s.[b,c]	598 ± 36.7 n.s.[b,c]
A_K	40.76 ± 6.42	556 ± 44.4	30.32 ± 4.85 n.s.[b,c]	573 ± 48.7 n.s.[b,c]
18-OH DOC	11.13 ± 2.32	274 ± 36.9	15.20 ± 3.79 n.s.[b,c]	375 ± 28.0 n.s.[b,c]
18-OH B_K	10.69 ± 1.74	478 ± 26.8	24.37 ± 3.96 $p = 0.05$[c]	521 ± 64.0 n.s.[b,c]
Aldosterone	6.21 ± 2.10	334 ± 33.0	8.64 ± 0.94 n.s.[b,c]	398 ± 16.3 n.s.[b,c]

[a] Each figure represents the mean ± SE for 4–7 incubations.
[b] n.s. = not significant.
[c] Compares prog and preg of same group.

logical because the specific activities of the metabolites should decrease the further removed they are from their precursor.

There was also a decrease in specific activities as follows: DOC → B_K → A_K → 18-OH B_K → aldosterone → 18-OH DOC (Table I). According to the above sequence, the immediate precursor of aldosterone would be 18-OH B_K. Also, since the specific activity of 18-OH DOC is lower than that of aldosterone, it can not be considered a precursor of aldosterone. However, it could be considered as an intermediate entering via a convergent pathway.

Experiment II: Effects of incubating adrenal glands from rats with 4-^{14}C-pregnenolone (S.A. = 1442 cpm/nmole) or 4-^{14}C-progesterone (S.A. = 1424 cpm/nmole).

The rat adrenal gland steroid production in nanomoles and specific activities are depicted in Table II. All steroid values have been corrected for losses that occurred during their isolation and characterization which were usually of the same magnitude as previously stated. The combined adrenal gland weights were 29.1 ± 1.9 mg, whereas the range per incubation was 9–16.2 mg.

Both the total amount and specific activity of DOC from progesterone was significantly higher than from pregnenolone (Table II). Again, there was an apparent trend for the above compounds to have a higher quantity and specific activity from progesterone than from pregnenolone. Also, in all cases the specific activities decreased from DOC to aldosterone. As in the rabbit experiments, the fact that pregnenolone is further removed in the biosynthetic scheme than progesterone should be taken into consideration when observing the tables. The specific activity of 18-OH B_K was in all cases the nearest to the value of aldosterone.

Discussion. By the data presented in this work, it is not possible to resolve the exact pathway for the formation of aldosterone in either the rat or rabbit adrenals. If one considers specific activity data there seems to be little doubt that 18-OH B_K was the im-

TABLE II. Rat Adrenal Steroid Production (nmole/2 hr of incubation) and Steroid Specific Activity (cpm/nmole) Following Adrenal Gland Incubations with 4-^{14}C-pregnenolone (4-^{14}C-preg, S.A. = 1442 cpm/nmole) or 4-^{14}C-progesterone (4-^{14}C-prog, S.A. = 1424 cpm/nmole).[a]

	4-^{14}C-preg		4-^{14}C-prog	
	nanomoles	S.A.	nanomoles	S.A.
DOC	7.42 ± 0.69	851 ± 30.5	14.80 ± 2.10 $p = 0.025$[b]	1071 ± 26.9 $p = 0.01$[b]
B_K	11.84 ± 1.05	725 ± 36.5	14.90 ± 2.70 n.s.[b,c]	810 ± 38.5 n.s.[b,c]
18-OH DOC	4.88 ± 0.44	634 ± 25.3	6.26 ± 0.92 n.s.[b,c]	642 ± 24.5 n.s.[b,c]
18-OH B_K	2.03 ± 0.24	518 ± 15.0	2.89 ± 0.25 n.s.[b,c]	582 ± 33.4 n.s.[b,c]
Aldosterone	1.81 ± 0.27	421 ± 28.0	2.44 ± 0.26 n.s.[b,c]	444 ± 26.5 n.s.[b,c]

[a] Each figure represents the mean ± SE for 4–6 incubations.
[b] Compares prog and preg of same group.
[c] n.s. = not significant.

mediate precursor of aldosterone in both animals. Nicolis and Vlick (11) studied the formation of aldosterone in bullfrog, beef, and human adrenal slices from progesterone, DOC, B_K, and their corresponding 18-hydroxylated forms. Small but significant conversions of the 18-hydroxylated forms to aldosterone were reported. However, the 18-deoxysteroids were more effective precursors of aldosterone than the corresponding 18-hydroxysteroids. Sandor and Lanthier (23) and Stachenko and Giroud (24) also found higher conversions to aldosterone from progesterone and B_K than their 18-hydroxy derivatives. According to Nicolis and Ulick (11), the more rapid formation of aldosterone from the 18-deoxy precursors suggested the possibility of an alternate biosynthetic pathway not involving 18-hydroxylation. Such a pathway, however, would require an unlikely mechanism not previously described, the one-step biological oxidation of a methyl group to an aldehyde. The only known mechanism for the biosynthesis of a carbonyl from a methyl or methylene group involves a two-step process in which hydroxylation is followed by dehydrogenation. Other steroid carbonyls whose mode of biosynthesis is known, are formed in this manner.

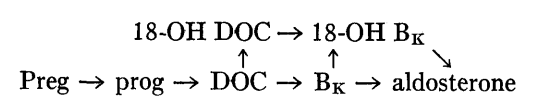
An alternate explanation, given by Nicolis and Ulick for the slower conversion of 18-hydroxylated steroids to aldosterone, is that these steroids exist in solution mainly in the cyclic 18, 20 hemiketal form as shown by infrared spectroscopy. The cyclic form is more resistant to chemical oxidation and may be more resistant to enzymatic dehydrogenation as well. Although the cyclic hemiketal is the predominant form added to the incubation medium, it is not necessarily the form produced at the enzyme surface when the angular methyl group undergoes 18-hydroxylation.

Later, Fazekas and co-workers (15–18) reported the conversion of A_K to aldosterone via 18-OH A_K by rabbit adrenal tissue. A compound isolated in the present experiment was tentatively identified as 18-OH A_K. Since the greater part of this compound was lost in the process of identification, its role in the formation of aldosterone could not be assessed. However, the relatively large amount of the 11-dehydro forms of B_K and F_K (A_K and E_K respectively) tend to emphasize the importance of the 11α OH-dehydrogenase activity in the rabbit adrenal. This was not a characteristic of the rat adrenal under the conditions of this experiment.

Cortes, Peron, and Dorfman (25) presented evidence that 18-OH DOC in the rat is controlled at least in part by ACTH. Al-

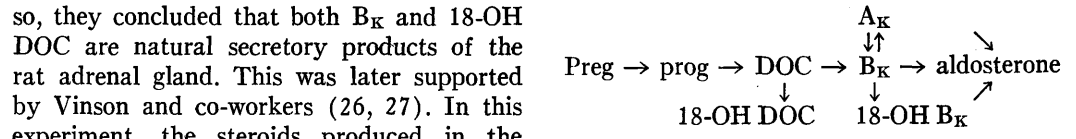
so, they concluded that both B_K and 18-OH DOC are natural secretory products of the rat adrenal gland. This was later supported by Vinson and co-workers (26, 27). In this experiment, the steroids produced in the largest amounts in the rat adrenal gland were B_K, DOC, and 18-OH DOC. This was consistent with the data of the above investigators.

If it is accepted that the specific activities of the metabolites will decrease the further removed they are from their precursor, then specific activity data can be used for constructing biosynthetic pathways. The compounds, B_K, 18-OH DOC, and 18-OH B_K, were all possible precursors of aldosterone in the rat adrenal gland. In the rat adrenal gland, pregnenolone and progesterone were transformed as follows:



In most cases in rat adrenal incubations, 18-OH DOC had a specific activity comparable to B_K, and both had a specific activity less than DOC. This suggested their position in the biosynthetic scheme as shown above. The specific activity of 18-OH B_K under all conditions of this experiment was between the above two compounds and aldosterone. Using specific activity data as a criteria of product–precursor relationship, 18-OH B_K was the immediate precursor of aldosterone.

In considering the data on the formation of aldosterone in the rabbit adrenal, there are some apparent differences from that of the rat adrenal gland. There was more 18-OH B_K formed than 18-OH DOC in the rabbit adrenal gland. A puzzling observation in the rabbit experiments is that 18-OH DOC had a lower specific activity than aldosterone. This was interpreted as proof that 18-OH DOC was not a precursor of aldosterone in the rabbit adrenal gland. In the rabbit 18-OH B_K, B_K, and A_K were all possible precursors of aldosterone. Pregnenolone and progesterone were transformed to aldosterone in the rabbit adrenal with the possible intermediates as follows:



As in the rat adrenal, the specific activity of 18-OH B_K would place it as the immediate precursor of aldosterone in the rabbit adrenal gland.

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