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# PATHWAYS FOR *IN VITRO* BIOSYNTHESIS OF CORTICOSTERONE AND CORTISOL IN THE GUINEA-PIG ADRENALS*

VASANT V. PATWARDHAN and ANDRÉ LANTHIER
Laboratoire d'Endocrinologie, Hôpital Notre-Dame et Département de Médecine, Université de Montréal, Québec, Canada

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SUMMARY

Quarters of guinea-pig adrenals were incubated in the presence of radioactive pregnenolone and progesterone (bearing different labels) and doubly labelled progesterone, 17-hydroxyprogesterone, corticosterone and cortisol were isolated from the incubates. The pregnenolone isotope/progesterone isotope ratios in these compounds were then used to interpret the operative pathways for the formation of corticosteroids. These experiments were done under three different conditions: (1) the two C-21 steroid substrates were made isomolar before incubation, (2) the substrates were non-isomolar and (3) preincubated adrenal tissue was used. Under all the three conditions it was observed that in the guinea-pig adrenals progesterone may not be an obligatory intermediate for corticosterone biosynthesis. Further, cortisol has seen to be formed by a pathway bypassing both progesterone and 17-hydroxyprogesterone.

## INTRODUCTION

Pathways for the biosynthesis of corticosteroids have been investigated in detail by use of *in vitro* incubation and perfusion techniques. It is generally accepted[1] that the normally operative pathway for the biosynthesis of corticosterone from pregnenolone in the adrenal glands is through progesterone. However, some species differences have been noted in this regard. Thus, in human[2], bovine[3] and rat[4] adrenals corticosterone formation from pregnenolone has been shown to proceed *via* progesterone. In other species like the sheep[5], the duck and the chicken[6] progesterone may not be an obligatory intermediate for corticosterone biosynthesis. In the cortisol producing species like the human, pregnenolone has been shown to be converted to cortisol by the adrenals by a route bypassing progesterone. While 17-hydroxypregnenolone has been established as an intermediary in this respect, the role of 17-hydroxyprogesterone (17OHP) is in some doubt and it is thought that the biosynthetic route for cortisol can bypass 17OHP[7] on its way to 11-deoxycortisol. In the case of the bovine adrenals, however, we have found that in this species cortisol is formed from pregnenolone mainly *via* 17OHP[8].

In view of such species differences in the pathways for adrenal biosynthesis of corticosterone and cortisol we carried out some experiments to see if in the guinea-pig adrenals[1] progesterone is an obligatory precursor for biosynthesis of corticosterone and[2] if progesterone and 17OHP are intermediates in cortisol biosynthesis. For this purpose quarters of guinea-pig adrenals were incubated in the presence of labelled pregnenolone and progesterone (bearing different isotope labels) and doubly labelled progesterone, 17OHP, corticosterone and cortisol were isolated from the incubates. The isotope ratios in these compounds were then used to interpret the operative pathways for the formation of corticosteroids. These experiments were done under three conditions: (1) the two C-21 steroid substrates were made isomolar before incubation (2) the stubstrates were non-isomolar and (3) the adrenals were preincubated in the incubation buffer before using the tissue.

Our results indicate that in the guinea-pig adrenals progesterone may not be an obligatory intermediate for corticosterone biosynthesis and that cortisol can be formed by a pathway bypassing both progesterone and 17-OHP. We wish to report here these results.

## MATERIALS AND METHODS

*Radioactive compounds.* Labelled pregnenolone and progesterone were obtained from New England Nuclear, Boston, U.S.A. and purified before use by paper chromatography.

*Reagents and solvents.* All steroids used in the experiments were crystallized before use. Reagents and solvents were of analytical grade.

* The following trivial names and abbreviations have been used in the text: corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; cortisol, 11β,17,21-trihydroxy-4-pregnene-3,20-dione; dehydroepiandrosterone, 3β-hydroxy-5-andosten-17-one; 11-deoxycortisol, 17,21-dihydroxy-4-pregnene-3,20-dione; 17-hydroxypregnenolone; 3β,17-dihydroxy-5-pregnen-20-one; 17-hydroxyprogesterone (17OHP), 17-hydroxy-4-pregnene-3,20-dione; pregnenolone, 3β-hydroxy-5-pregnen-20-one; progesterone, 4-pregnene-3,20-dione.

*Detection and counting of radioactivity.* Radioactivity on paper and thin-layer plates was detected with the aid of a radio-chromatogram scanner (Model 7200, Packard Instrument Co., Downer's Grove, Ill.). Counting of $^{3}H$ and $^{14}C$ content of samples was done on a three-channel liquid scintillation spectrometer (Tri-Carb, Model 3375, Packard).

*Incubations.* Adult white male guinea pigs were sacrificed by decapitation. The adrenals were removed and freed of the extraneous tissue. Each adrenal gland was cut into four approximately equal pieces. Two of these pieces were randomly selected for group A while the other two pieces formed part of group B. For an experiment, adrenals from six animals were used, each adrenal contributing to the two pooled tissue groups A and B as described before.

(A) *Incubation with non-pre-incubated adrenals.* Tissue in group A was weighed and put in a beaker containing 5 ml of Krebs–Ringer bicarbonate buffer, pH 7·4, 20 mg glucose and labelled pregnenolone and progesterone as substrates. (The steroids had been solubilized in 0·2 ml propylene glycol). Details of the nature and amount of substrates used are shown in Tables 1 and 2. The beaker was then incubated in a Dubnoff metabolic shaking incubator at 37° in an atmosphere of 95% $O_2$ and 5% $CO_2$ for 3 h and the incubation terminated by addition of acetone.

(B) *Incubation with pre-incubated adrenals.* Tissue in group B was weighed and put in a beaker containing 5 ml of Krebs–Ringer bicarbonate buffer and incubated in the Dubnoff incubator for 45 min. The beaker was then removed from the bath, the medium was decanted and the tissue washed once with 5 ml of fresh Krebs–Ringer buffer, the wash being rejected. The tissue was then added to another beaker containing an identical medium of buffer and labelled C-21 steroids as used for group A. It was incubated for 3 h at 37° under 95% $O_2$ and 5% $CO_2$ and the incubation terminated by addition of acetone.

Two such experiments were done. In Experiment I the substrates were made isomolar while in Experiment II they were non-isomolar (Tables 1 and 2).

Table 1. Experiment I: Pregnenolone isotope/ progesterone isotope ratios of compounds isolated after the simultaneous incubation of [7α-$^{3}H$]-pregnenolone ($3{\cdot}24 \times 10^{6}$ d.p.m. S.A. 20 Ci/mmol) and [4-$^{14}C$]-progesterone ($4{\cdot}32 \times 10^{6}$ d.p.m. S.A. 52·8 mCi/mmol) with pieces of guinea-pig adrenals (substrates were made isomolar)

| Nature of tissue used | Pregnenolone isotope/progesterone isotope ratios | | | |
|---|---|---|---|---|
| | Prog-esterone | 17-OHP | Corti-costerone | Cortisol |
| (A) Without pre-incubation | 0·338 | 0·392 | 0·581 | 0·590 |
| (B) Pre-incubated | 0·081 | 0·396 | 0·543 | 0·584 |

Table 2. Experiment II: Pregnenolone isotope/ progesterone isotope ratios of compounds isolated after the simultaneous incubation of [4-$^{14}C$] pregnenolone ($1{\cdot}91 \times 10^{6}$ d.p.m. S.A. 52·4 mCi/mmol) and [7α-$^{3}H$] progesterone ($1{\cdot}78 \times 10^{6}$ d.p.m. S.A. 20 Ci/mmol) with pieces of guinea-pig adrenals (substrates were not isomolar and the incubated mass was: 5·24 μg and 0·012 μg respectively)

| Nature of tissue used | Pregnenolone isotope/progesterone isotope ratios | | | |
|---|---|---|---|---|
| | Prog-esterone | 17-OHP | Corti-costerone | Cortisol |
| (A) Without pre-incubation | 0·456 | 0·331 | 1·056 | 1·17 |
| (B) Pre-incubated | 0·158 | 0·391 | 1·211 | 1·60 |

*Extraction, isolation and identification of compounds.* From each incubate, doubly labelled radioactive progesterone, 17-OHP, cortisol and corticosterone were isolated. Before extraction, 100 μg each of the non-radioactive steroids (mentioned above) was added to an incubate to facilitate the isolation procedure. Extraction of the incubates was done with alcohol–acetone (1 : 1 v/v) mixture. The isolation of progesterone, 17-OHP, cortisol and corticosterone from this extract was achieved following multiple paper and silica gel thin-layer chromatographies coupled with molecule modifying reactions such as acetylation and chromic acid oxidation. Procedures followed in the above mentioned techniques have been described before in our previous publications [3, 8]. The isolated fractions were crystallized to constant specific activity after addition of the appropriate carrier steroid.

## RESULTS AND DISCUSSION

Pregnenolone isotope/progesterone isotope ratios for $^{14}C$ and $^{3}H$ labelled progesterone, 17-OHP, corticosterone and cortisol isolated from the incubations of Experiments I and II are shown in Tables 1 and 2. Crystallization data on compounds isolated in Experiment I are shown in Table 3. Compounds isolated in Experiment II were also crystallized to constant specific activity and were of similar radiochemical purity. We may add that both the experiments were carried out on pooled adrenal tissue and a statistical analysis of the results was not possible. However, the trend of the isotope ratios in different compounds discussed below was the same under three different experimental conditions and led to the same conclusions.

Although the radioactive label from both pregnenolone and progesterone was incorporated into corticosterone and cortisol, pregnenolone is the earlier precursor of these compounds. Therefore, the pregnenolone isotope/progesterone isotope

Table 3. Data on crystallization of doubly labelled progesterone, 17-hydroxyprogesterone, cortisol and corticosterone isolated in Experiment I after incubation of guinea-pig adrenals with [7α-$^3$H]-pregnenolone ($3{\cdot}24 \times 10^6$ d.p.m. S.A. 20 Ci/mmol) and [4-$^{14}$C] progesterone ($4{\cdot}32 \times 10^6$ d.p.m. S.A. 52·8 mCi/mmol). Substrates were made isomolar

| Compound | Isolated and crystallized as | Pool S.A. d.p.m./mg | | Crystals S.A. d.p.m./mg | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | n-2 | | n-1 | | n+ | |
| | | $^3$H | $^{14}$C | $^3$H | $^{14}$C | $^3$H | $^{14}$C | $^3$H | $^{14}$C |
| Progesterone | Progesterone | | | | | | | | |
| A* | | 28457 | 77521 | 24328 | 71630 | 24605 | 72357 | 24352 | 72462 |
| B | | 7111 | 73755 | 5267 | 64070 | 4862 | 62161 | 5086 | 62339 |
| 17-hydroxy progesterone | Androstenedione | | | | | | | | |
| A | | 1223 | 2832 | 1092 | 2736 | 1097 | 2752 | 1049 | 2750 |
| B | | 842 | 1972 | 781 | 1976 | 779 | 1926 | 767 | 1963 |
| Cortisol | Cortisol acetate | | | | | | | | |
| A | | 1596 | 2654 | 1478 | 2506 | 1536 | 2589 | 1492 | 2532 |
| B | | 2924 | 4995 | 2965 | 4941 | 2860 | 4752 | 2703 | 4900 |
| Corticosterone | 11-Dehydrocorti-costerone acetate | | | | | | | | |
| A | | 9491 | 16372 | 8668 | 15440 | 8679 | 14646 | 8429 | 14241 |
| B | | 6155 | 11051 | 4763 | 8994 | 4770 | 8806 | 4781 | 8563 |

n+ Represents the number of crystallizations and usually was equal to 4.
A*) Compound isolated from incubation of non-preincubated tissue.
B) Compound isolated from incubation of preincubated tissue.

ratios of the final products corticosterone and cortisol should be less than or at the most equal to those of the postulated intermediates (progesterone or 17-OHP). However, a comparison of the isotope ratios of progesterone and corticosterone isolated in our experiments using non-pre-incubated guinea-pig adrenals (Section A, Tables 1 and 2) shows that the pregnenolone isotope/progesterone isotope ratios in corticosterone are higher than those in the postulated intermediate, progesterone. We interpret this observation to mean that the major operative pathway from pregnenolone to corticosterone was not derived through progesterone, although the latter pathway may have been operative also. Similarly, the isotope ratios in cortisol are higher than in both progesterone and 17-OHP indicating that a significant portion of pregnenolone was converted to cortisol by a pathway bypassing progesterone and 17-OHP. The trend in isotope ratios discussed above was more marked in compounds isolated from Experiment II (Table 2, A) than in those from Experiment I (Table 1, A). This could have been due to the differences in substrate concentrations used in the experiments. In Experiment I, the two steroid substrates were made isomolar while in experiment II there was an excess of pregnenolone substrate (5·24 μg) over the progesterone substrate (0·012 μg). It may, however, be mentioned that unutilized substrates were recovered in both the experiments.

Thus our experiments indicate that in the guinea-pig, as in certain other species like the sheep[5], the duck and the chicken[6], progesterone may not be an obligatory intermediate for corticosterone biosynthesis.

Deshpande *et al.*[9] have previously studied cortisol and androgen biosynthesis in guinea-pig adrenals using a continuous *in vivo* infusion technique. After infusing a combined dose of [$^3$H]-17-hydroxypregnenolone and [$^{14}$C]-progesterone they found that the major pathway to cortisol was *via* 17-hydroxypregnenolone and not *via* progesterone. We have now observed that the major pathway may also bypass 17-OHP. We should like to point out that the results of our experiments are of indicative and not definitive nature. This is because as in other *in vitro* studies of similar type[10–12] the interpretation of results is limited by the fact that factors like the endogenous concentration of the various steroids in the incubated tissue and the possible different penetration of the two substrates through membranes have not been taken into account.

Many investigators use tissue from pre-incubated adrenals for *in vitro* studies. It was therefore of interest to see if there was a difference between tissues obtained from non-pre-incubated and pre-incubated adrenals with regard to the biosynthetic pathways for corticosteroids. A comparison of the isotope ratios in progesterone isolated from the pre-incubated and non-pre-incubated adrenal incubations (Tables 1 and 2) shows that pre-incubation of the adrenals resulted in a marked loss of 3β-hydroxysteroid dehydrogenase-isomerase reaction in that tissue. Tsang and Carballeira[13] have previously observed that after incubation of bovine or rat adrenals there was a leakage of the enzyme complex in the medium. This may partly explain our observation. It is also possible that there might have been a loss of cofactors required for the 3β-hydroxysteroid dehydrogenase-isomerase reaction. However, the conclusions arrived at about the relatively minor role of progesterone and 17-OHP in corticosteroid biosynthesis in the guinea-pig adren-

als using non-pre-incubated tissue are essentially valid also in the case of experiments using tissue from pre-incubated adrenals (Section B, Tables 1 and 2). The discrepancy in the isotope ratios of progesterone and corticosterone was much more marked in this case because of decreased rate of conversion of pregnenolone to progesterone.

Attempts were made to isolate radiolabelled dehydroepiandrosterone from the incubates. However, no significant amount of radioactivity was found to be associated with isolated dehydroepiandrosterone fractions. It may be mentioned that Deshpande *et al.*[9] did not find conclusive evidence for synthesis of dehydroepiandrosterone or its sulphate after *in vivo* infusion of pregnenolone or 17-hydroxypregnenolone. The results of our experiments and those of Deshpande *et al.* are in contrast with those of Trout *et al.*[14] who found that homogenates from guinea-pig adrenals converted 17-hydroxypregnenolone to dehydroepiandrosterone.

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