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# *IN VITRO* STUDY ON CORTICOSTERONE CYTOCHROME P-450 BINDING IN RELATION TO THE REGULATION OF CORTICOSTERONE METABOLISM

J.-G. LEHOUX and J. C. FOREST

Département d'Obstétrique et Gynécologie, Université de Sherbrooke, Sherbrooke, Canada J1H 5N4

## SUMMARY

The effects of various factors were studied in relation to the *in vitro* corticosterone metabolism and corticosterone binding by beef adrenocortical cytochrome P–450 preparations. Temperature increments (0 37°C) enhanced the transformation of corticosterone to aldosterone and the binding of corticosterone to cytochrome P–450. pH lower than 7·0 and higher than 7·5 and increasing urea molarities of media diminished the corticosterone-cytochrome P-450 binding values and the transformation of corticosterone to aldosterone. High concentration of sodium and reduction of the media with sodium hydrosulfite provoked alterations of corticosterone association to cytochrome P–450 and inhibited the transformation of corticosterone to aldosterone. In our opinion the factors studied could play an important role in the corticosterone metabolism rate by influencing the binding of corticosterone to the enzyme cytochrome P–450.

## INTRODUCTION

The conversion of corticosterone (B) to aldosterone (aldo) appears to be an important step in the regulation of mineralocorticosteroid synthesis by adrenal glands. Indeed, it has been reported that potassium and ACTH enhanced the *in vitro* transformation of B to aldo in rat adrenal glomerulosa tissue[1, 2]. Capsular adrenal glands of potassium deficient rats converted much less B to aldo than controls[3]. Sodium depletion, on the other hand, increased the rate of this reaction[4], while sodium intake reduced it[5, 6]. Further *in vitro* studies have demonstrated that the conversion of B to aldo was inhibited by puromycin[7].

The present investigation was designed to examine some aspects of the mechanism of control of aldosterone biosynthesis. Corticosterone, the precursor of aldosterone, binds to cytochrome P-450 (P-450), which is a component of the 18-hydroxylating system[8, 9], causing characteristic changes in optical absorbance. It appeared pertinent to us to investigate the effect on the spectral properties of corticosterone-P-450 binding complex by factors known to affect the transformation of corticosterone to aldosterone by adrenal tissue. In this paper, we report the results from an investigation of the effect of ion concentration, temperature, pH, urea, and puromycin on the binding of B to P-450 in relation to the enzymatic conversion of B to aldo.

## MATERIALS AND METHODS

### *Adrenal tissue*

Beef adrenals were obtained as soon as possible after slaughter. Adhering fat, medulla, fasciculata and reticulata zones were carefully removed and the glomerulosa rich fraction was homogenized with twice its weight of ice-cold 0·25 M sucrose solution buffered to pH 7·0 with Tris–HCl 0·05 M. The mitochondrial fraction obtained after centrifugation at 6500 *g* was washed with the sucrose solution and recentrifuged at 6500 *g* for 10 min. This latter precipitate was suspended in Tris–HCl 0·05 M and used without any further purification.

### *Incubation*

Incubations were performed at 37° for 20 min in a Dubnoff metabolic shaker. Incubation medium was buffered to pH 7·0 with Tris–HCl 0·05 M and contained $MgCl_2$ 8·5 mM, $CaCl_2$ 2·7 mM, KCl 3·13 mM, NaCl 7·59 mM, bovine albumin 2% and NADPH 0·5 mM as cofactor in a total volume of 3 ml. The substrate, [$^3$H]-corticosterone (1 $\mu$Ci), was added to the incubation vials in 0·05 ml of ethylene glycol before any other addition. After incubation, the contents of the incubation vial were transferred to a separation funnel with known amounts of [$^{14}$C]-aldosterone to monitor losses occurring during isolation manipulations; extractions were then performed with chloroform: ethylacetate (1 : 1 v/v).

*Purification and characterization procedures*

The biosynthesized aldosterone was purified by paper partition chromatography and derivative formation as previously described by Sandor and Lanthier [10].

*Substrates and other chemicals*

$^{3}$H-corticosterone (specific activity 50 Ci/mmol and [$^{14}$C]-aldosterone (specific activity 55 mCi/mmol were obtained from New England Nuclear Co.: non radioactive corticosterone, aldosterone, were purchased from Ikapharm Co. Puromycin was obtained from Sigma Co. and sodium hydrosulfite from Fisher Co.

*Spectrophotometric studies on mitochondrial cytochrome* P-450.

Spectra were obtained using a Unicam SP-800 recording spectrophotometer equipped with temperature controlled cell holder. Most spectra were taken at 0°C. Mitochondria were suspended in Tris–HCl 0·05 M pH 7·0 and difference spectra induced by the binding of substrates to cytochrome P-450 were recorded by scanning between 370 nm and 500 nm.

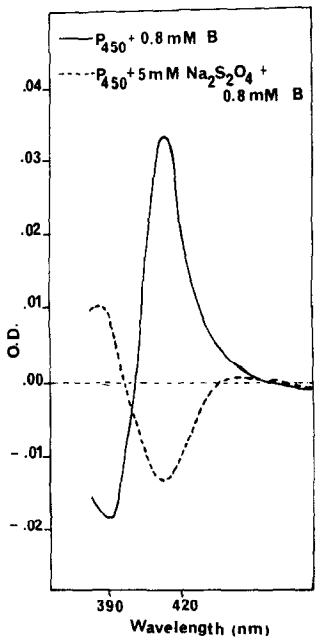


Fig. 1. Difference spectra of the corticosterone P-450 complex in a non-reduced preparation (straight line) and a sodium hydrosulfite-reduced preparation (dotted line). Mitochondria were suspended in 1·1 mg/ml Tris–HCl 0·05 M, pH 7·0 and divided between two cuvettes with 1 cm lightpath. The sample cuvette contained 0·8 mM corticosterone. In a second experiment (dotted line) $Na_2S_2O_4$ was added to each cuvette prior to the addition of corticosterone. The difference spectrum was recorded at 0°C.

After precipitation with trichloroacetic acid, proteins were determined by the technique of Lowry[11] using bovine serum albumin as standard.

## RESULTS

*Effect of reduction*

The effect of the reduction of mitochondrial preparation by sodium hydrosulfite on the binding of B to P-450 was studied. Figure 1 shows the type of difference spectra induced by the addition of 0·8 mM of B to the non reduced preparation (straight line curve); this curve presents a maximum at about 420 nm and a minimum at about 390 nm typical of inverted type I induced difference spectra according to Schenkman *et al.*[12]. The addition of sodium hydrosulfite (5 mM) to the preparation resulted in the inversion of this spectrum to produce a new spectrum with a maximum at about 390 nm and a minimum at about 420 nm, typical of type I induced difference spectra (dotted line, Fig. 1). The addition of sodium hydrosulfite from 0 to 5 mM resulted in the gradual inversion of the spectrum.

These results suggest that variation of the oxido-reduction state of the enzyme produces an alteration in the enzyme–substrate association. Oxidation of P-450 with hydrogen peroxide reversed this situation and regenerated the reversed type I spectrum.

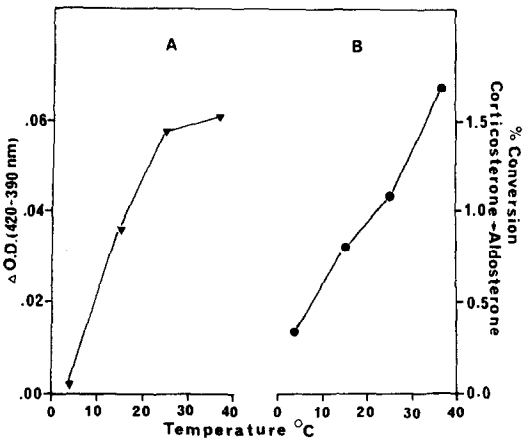


Fig. 2. Effect of temperature on corticosterone binding to P-450 (A) and on the enzymic conversion of corticosterone to aldosterone (B) by mitochondrial preparations from beef adrenal glomerulosa. (A) Mitochondria were suspended to 1·2 mg/ml Tris–HC10·05 M, pH 7·0 and divided between two cuvettes. The sample cuvette contained 0·2 mM corticosterone and the difference in optical density between 420 nm and 390 nm (ΔO.D. 420–390 nm) was recorded. (B) Mitochondria (1 mg/3 ml Tris–HCl 0·05 M, pH 7·0, final volume) were incubated for 20 min in the presence of 1 $\mu$Ci of [$^{3}$H]-corticosterone and 0·5 mM NADPH. The results are expressed in terms of the percentage of [$^{3}$H]-B transformed to [$^{3}$H]-aldosterone.

Table 1. Effect of urea on the binding of B to P-450 and on the conversion of [$^3$H]-B to [$^3$H]-aldo. (A) Mitochondria were suspended in TRIS-HCl 0·05 M, pH 7 (2.2 mg prot./ml) and divided into pairs of cuvettes. Urea was added at various concentrations to each pair of cuvettes and corticosterone (0·8 mM) was added to each sample cuvette. Difference in optical density between 420 and 390 nm was recorded at 0°C. (B) Mitochondria (2.25 mg prot./3 ml TRIS-HCl 0·05 M, pH 7·0) were incubated with 1 $\mu$Ci [$^3$H]-B and 0·5 mM NADPH for 20 min at 37°C. Results are expressed in terms of the percentage of [$^3$H]-corticosterone transformed into [$^3$H]-aldosterone.

| Urea conc. (M) | Δ O.D. (420-390 nm) | % Conversion B→Aldo. |
|---|---|---|
| 0.0 | .100 | .90 |
| 1.0 | .050 | .70 |
| 1.5 | .037 | .44 |
| 2.0 | .022 | .26 |

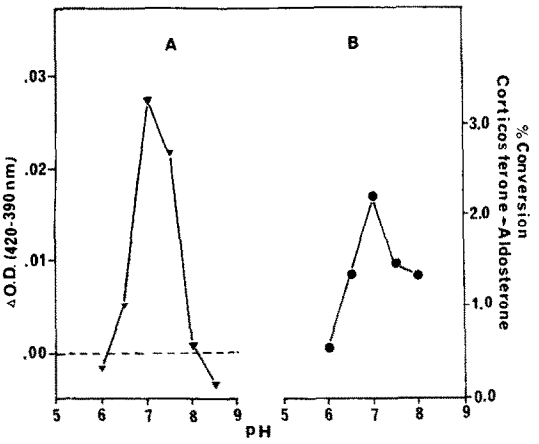


Fig. 3. Effect of pH on corticosterone binding to P-450 (A) and on the enzymic conversion of corticosterone to aldosterone (B) by mitochondrial preparations from beef adrenal glomerulosa. (A) Mitochondria were suspended to 1·5 mg/ml Tris–HCl 0·05 M and divided between two cuvettes. The sample cuvette contained 0·8 mM corticosterone and the difference in optical density between 420 and 390 nm was recorded at 0°C. (B) Mitochondria (1 mg/3 ml Tris-HCl 0·05 M, final volume) were incubated for 20 min in the presence of 1 $\mu$Ci [$^3$H]-corticosterone and 0·5 mM NADPH. Results are expressed in terms of the percentage of [$^3$H]-B transformed to [$^3$H]-aldosterone.

*Effect of temperature*

The effect of temperature was also studied. The difference in optical density (420–390 nm) produced by the addition of corticosterone to mitochondrial cytochrome P-450 preparations was used as a B-P-450 binding index value. As seen in Fig. 2 (A), increments of temperature from 4°C to 37°C produced a nearly six fold increase of the binding index value. The curve (B) of Fig. 2 shows that temperature increments also enhanced more than five times the percentage of transformation of [$^3$H]-corticosterone to [$^3$H]-aldosterone.

*Effect of urea*

The addition of increasing concentrations of urea to mitochondrial cytochrome P-450 preparations diminished progressively the B-P-450 index value as shown in Table 1. Table 1 shows results obtained on the transformation of [$^3$H]-corticosterone to [$^3$H]-aldosterone in the presence of increasing concentrations of urea in the incubation media. It can be seen that the percentage of B metabolism was diminished considerably in the presence of urea. These results show a good correlation with those of spectral experiments shown on the left column of Table 1.

*Effect of pH*

The pH of the medium has an influence in the type of spectrum produced by the association of B to P-450. At pH 6·0 a type I spectrum was induced which became inverted at higher pH, reaching a maximum at physiological pH as shown in Fig. 3A. The curve B of Fig. 3 represents the results obtained from an experiment performed to study the effect of the pH variation on the transformation of [$^3$H]-corticosterone to [$^3$H]-aldosterone. It is shown that the highest conversion percentage occurred at pH 7·0 which gave also the optimal B-P-450 association index.

*Effect of ionic composition of the medium*

We have studied the effect of sodium, potassium, calcium and EDTA on the binding of corticosterone to cytochrome P-450. As shown in Fig. 4 at low concentrations of corticosterone and at high concentrations of sodium there is a tendency towards the formation of type I difference spectra. At a concentration of 140 mM sodium, the corticosterone (0·8 mM) induced a type I spectrum equal only to 0·4 of the value obtained when no sodium was present in the medium. This suggests that sodium could interfere directly to inhibit the binding of corticosterone to cytochrome P-450 and consequently interfere with the transformation of corticosterone to aldosterone. Potassium and calcium at concentrations from 0 to 10 mM and EDTA (1 mM) did not affect significantly the binding of corticosterone to cytochrome P-450.

*Effect of puromycin*

Marusic and Mulrow[7] have recently shown that

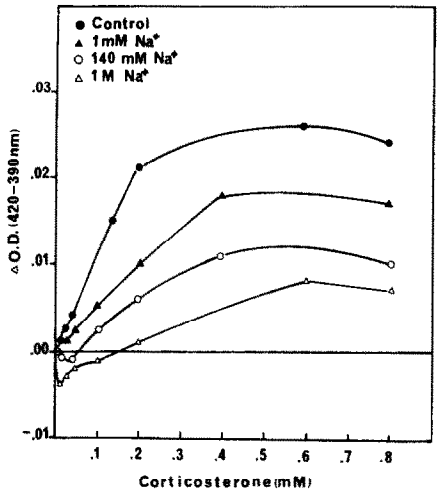


Fig. 4. Effect of various concentrations of sodium on corticosterone binding to P-450. Difference in optical density between 420 nm and 390 nm was recorded at 0°C with increasing concentrations of corticosterone. Mitochondria were suspended to 1·0 mg protein/ml Tris–HCl 0·05 M pH 7·0 and corticosterone and sodium chloride were added to the sample cuvette.

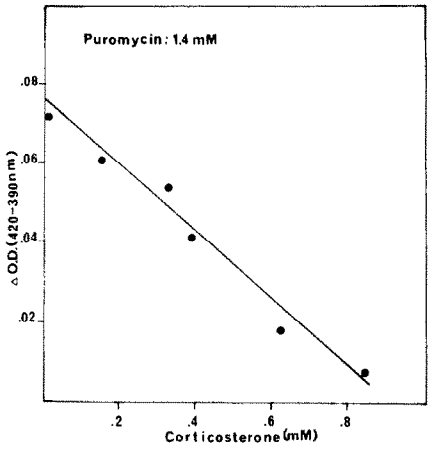


Fig. 6. Inhibition of puromycin binding to P-450 by increasing concentrations of corticosterone. Mitochondria were suspended in Tris–HCl buffer 0·05 M, pH 7 to a protein concentration of 1·2 mg/ml and divided into two cuvettes. Mitochondria preparations of both cuvettes were preincubated with corticosterone and 1·4 mM puromycin was added to the sample cuvette. Difference in optical density between 420 and 390 nm were recorded at 0°C.

puromycin at a concentration of 10 mM inhibited the conversion of corticosterone to aldosterone. Their data are consistent with the hypothesis that puromycin has a direct effect on the enzyme involved on the conversion of B to aldosterone. We thought it pertinent to study a possible interaction of puromycin with cytochrome P-450. Figure 5 shows the spectrum induced by the addition of puromycin (1·4 mM) to a preparation of P-450. This curve is very similar to the one observed when B is used as substrate (see Fig. 1) presenting a maximum at 420 nm and a minimum at 390 nm (inverted type I spectrum).

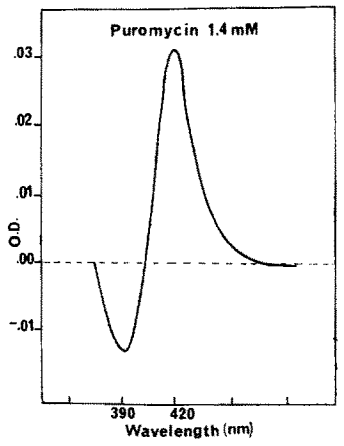


Fig. 5. Difference spectrum of puromycin P-450 complex. Mitochondria were suspended in Tris–HCl buffer 0·05 M pH 7·0 to a protein concentration of 1·2 mg/ml and divided into two cuvettes. The sample cuvette contained 1·4 mM puromycin. The difference spectrum was recorded at 0°C.

The competition between puromycin and B for binding sites on the P-450 molecule was studied. A pool of mitochondria containing 1·2 mg/ml of protein was divided in aliquots. Increasing concentrations of B were added to both the control and the sample cuvettes and preincubated. Then 1·4 mM of puromycin was added to the sample cuvettes and spectrophotometric analysis were performed. The sample cuvette was read against another cuvette containing the same concentration of B as the sample cuvette but no puromycin. The Fig. 6 shows that B prevented the binding of puromycin to P-450 and B can displace puromycin from binding sites. Puromycin, on the other hand, does not seem to displace B on P-450 (unshown results).

Experiments performed on the transformation of [$^3$H]-B to [$^3$H]-aldosterone confirm this finding. Puromycin at a concentration of ($10^{-3}$ to $10^{-4}$ M) inhibited the biochemical transformation by 40–50% but at concentrations of ($10^{-5}$ to $10^{-6}$ M) it had no inhibiting effect on [$^3$H]-aldosterone formation even when the inhibitor was preincubated with the enzyme preparation prior to the addition of corticosterone.

## DISCUSSION

From the present series of experiments it appears that factors contributing to the formation of inverted type I spectra (minimum at 390 nm and maximum at 420 nm) resulting from the binding of B to mito-

chondrial cytochrome P-450 also favoured the rapid conversion of B to aldo. Indeed increments in temperature up to 37°C and optimal pH (7·0) concomitantly favoured the production of inverted type I spectrum and a faster conversion of B to aldo. Furthermore, the reduction of P-450 by sodium hydrosulfite decreased the inverted type I binding of B to P-450 and favoured the appearance of typical type I spectra (minimum at 420 nm and maximum at 390 nm). Oxidation with hydrogen peroxide on the other hand increased the inverted type I spectrum of the B-P-450 complex. These observations are consistent with previous information that cytochrome P-450 should be in the oxidized state before binding the substrate. Indeed, in the case of the hydroxylation of deoxycorticosterone, Estabrook *et al.* [13] have postulated that the oxidation state of P-450 is a prerequisite for the binding of the substrate to P-450. Sweat *et al.*[14] have also reported that deoxycorticosterone binds to the oxidized form of P-450.

As shown in Table 1 high urea concentrations produced a diminution of the binding of B to P-450 and also a diminution of the quantity of B transformed to aldo. These observations do not easily correlate with the enzyme degradation since at low concentration and up to 2 M, urea did not degrade the cytochrome P-450 to the inactive P-450 form. However at a concentration of 4 M, urea degraded the cytochrome and no spectrum could be obtained by the addition of corticosterone to this preparation.

The fact that high concentrations of sodium reduced the intensity of the binding value (ΔO.D. 420–390 nm) of B to P-450 is in good agreement with *in vivo* observations[4, 6] that sodium loading results in the diminution of aldosterone secretion. Unfortunately *in vitro* data on the biochemical transformation of B to aldo under various sodium concentrations are not yet available.

To our knowledge it is the first time that puromycin has been reported to induce a spectrum in the presence of cytochrome P-450 which implies binding to the pigment. Other protein synthesis inhibitors such as cycloheximide and chloramphenicol did not induce any spectrum under similar conditions. Our results are in good agreement with those of Marusic and Mulrow [7] in that the inhibition by puromycin is the result of a direct action between cytochrome P-450 and puromycin and not by inhibition of mitochondrial protein synthesis. The fact that low concentrations of puromycin cannot inhibit this reaction might be explained by the observation that corticosterone can displace easily this inhibitor as seen with the spectrophotometric experiments. It is therefore possible that puromycin inhibition of the transformation of corticosterone to aldosterone by beef adrenal mitochondria is accomplished by competition at the cytochrome P-450 level. Further investigation is underway to fully characterize the type of competition involved. In conclusion, it is our opinion that the medium composition can play an important role on the *in vitro* corticosterone metabolism in interfering with or favouring the binding of corticosterone to cytochrome P-450.

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## DISCUSSION

*McKerns*:

Your observations are most interesting. It has also been shown, I believe by the group in Philadelphia in Britton Chance's lab, that puromycin inhibits the electron transport system in the mitochondria, and the utilization of malate, for example. Both these observations and yours have great implications because one of the theories about the action of ACTH and other trophic hormones is that there is a rate-limiting protein involved in the conversion of cholesterol to pregnenolone and this evidence is based on inhibitions of

steroidogenesis by puromycin, implying that a rate-limiting protein needs to be synthesized.

*Neher*:

You said that cycloheximide had no action on the shift or spectrum change, though cycloheximide also inhibits steroidogenesis; thus I wonder if you have tried to see if something like adenosine or puromycin amino nucleoside which no longer inhibits protein synthesis also shifts your spectrum.

*Lehoux*:

The only protein synthesis inhibitors we have tried are the three I have mentioned, puromycin, cycloheximide and chloroamphenicol.

*Neher*:

These may be different effects, here you have a mM range of puromycin but if you go lower, you have even a stimulating effect with inihibited steroidogenesis.

*Lehoux*:

Puromycin inhibits at $10^{-3}$ to $10^{-4}$ M. At $10^{-5}$ to $10^{-6}$ M I have repeated many experiments and sometimes it stimulates a little and sometimes not. These results show a direct 18-hydroxylase inhibitory effect at slightly higher concentration than that seen for protein synthesis inhibition suggesting that puromycin may interact at two different levels of stereogenesis: inhibition of a rate-limiting protein and inhibition of the 18-hydroxylase activity.

*Müller*:

I would just like to emphasize that changes in zona glomerulosa 18-hydroxylase provoked by alteration in sodium or potassium intake have nothing to do with ambient sodium or potassium concentrations of the mitochondria. If you take mitochondria of sodium or potassium deficient or sodium loaded animals and incubate them in exactly the same type of buffer with the same electrolyte composition you will still find the differences in 18-hydroxylase activity.

*Lehoux*:

I believe that the ionic composition of the medium also has an effect on this enzyme activity. Indeed, we have shown (Fig. 4) that there were differences in the spectrum induced in the presence of increasing amounts of sodium in the medium. In addition, high sodium concentrations in the incubation medium inhibited the transformation of corticosterone to aldosterone. These observations are also in good agreement with those of Satura, T. *et al.* (*J. clin. Invest.* **51** (1972) 2239–2245) who demonstrated that increase of sodium in the medium from 100 m-equiv./l to 142 m-equiv./l decreased the conversion of corticosterone to aldosterone by beef adrenal outer slices.

*Kuss*:

Did you look for temperature dependence of binding constants?

*Lehoux*

What we have observed with those binding experiments is that the amount of corticosterone needed to produce the same difference in optical density between 420 and 390 nm is much less at higher temperatures than at lower temperatures.