D. Y., and Rosenthal, O. (1966), *Arch. Biochem. Biophys. 117*, 660.
Omura, T., and Sato, R. (1964a), *J. Biol. Chem. 239*, 2370.
Omura, T., and Sato, R. (1964b), *J. Biol. Chem. 239*, 2379.
Omura, T., Sato, R., Cooper, D. Y., Rosenthal, O., and Estabrook, R. W. (1965), *Federation Proc. 24*, 1181.
Pumphrey, A. M., and Redfearn, E. R. (1960), *Biochem. J. 76*, 61.
Salach, J., Tisdale, H. D., Singer, T. P., and Badu, P. (1967), *Biochim. Biophys. Acta 143*, 281.
Sato, R., and Imai, Y. (1961), *Biochim. Biophys. Acta 42*, 164.
Sato, R., Nishibayashi, H., and Omura, T. (1962), *Biochim. Biophys. Acta 63*, 550.
Savard, K. (1953), *J. Biol. Chem. 202*, 457.
Sharma, D. C., Forchielli, E., and Dorfman, R. I. (1962), *J. Biol. Chem. 237*, 1495.
Sottocasa, G. L., and Crane, F. L. (1965), *Biochemistry 4*, 305.
Sweat, M. L., and Lipscomb, M. D. (1955), *J. Am. Chem. Soc. 77*, 5185.
Williams, C. H., Jr., Gibbs, R. H., and Kamin, H. (1959), *Biochim. Biophys. Acta 32*, 568.
Williamson, D. G., and O'Donnell, V. J. (1967a), *Can. J. Biochem. 45*, 340.
Williamson, D. G., and O'Donnell, V. J. (1967b), *Can. J. Biochem. 45*, 153.
Williamson, D. G., and O'Donnell, V. J. (1969), *Biochemistry 8*, 1300 (this issue; paper II).
Wilson, L. D., Nelson, D. H., and Harding, B. W. (1965), *Biochim. Biophys. Acta 99*, 391.
Wosilait, W. D. (1960), *J. Biol. Chem. 235*, 1196.

The Role of Cytochrome P-450 in the Mechanism of Inhibition of Steroid 11β-Hydroxylation by Dicumarol*

Denis G. Williamson and Vincent J. O'Donnell

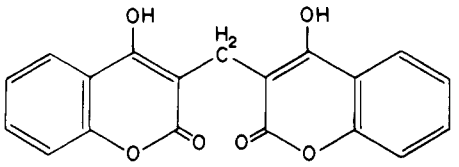
ABSTRACT: The mechanism of dicumarol inhibition of the 11β-hydroxylation of deoxycorticosterone has been examined in an 11β-hydroxylase system extracted from an acetone powder of beef adrenal mitochondria. At low concentrations of dicumarol the inhibition was noncompetitive with substrate deoxycorticosterone while inhibition at high concentrations of dicumarol was of a mixed type. Dicumarol inhibition of 11β-hydroxylation could be related to the effects of this compound on cytochrome P-450, the oxygen-activating and substrate-binding component of the 11β-hydroxylase system.

Cytochrome P-450 isolated from an acetone powder of beef adrenal mitochrondria was unstable, being rapidly converted into cytochrome P-420 during incubation at 37°. Addition of the steroid substrates deoxycorticosterone or androstenedione to the incubation mixture diminished both the extent and rate of decomposition of cytochrome P-450. The 11-oxygenated steroids, corticosterone and adrenosterone, did not have this effect. Dicumarol, like deoxycorticosterone, at low concentrations stabilized cytochrome P-450. The stabilizing effects of these two compounds were additive, a finding in agreement with the noncompetitive inhibition of 11β-hydroxylation by dicumarol. Dicumarol at high concentrations enhanced the rate of decomposition of cytochrome P-450. However, this effect was diminished by an increase in deoxycorticosterone concentration, suggesting that this inhibitory action of dicumarol was competitive with steroid substrate.

Recent studies have revealed that mitochondria of adrenocortical tissue possess an electron transport system, distinct from the classical respiratory chain, that is concerned with steroid 11β-hydroxylation (Omura *et al.*, 1965, 1966; Nakamura and Otsuka, 1966; Harding and Nelson, 1966; Cammer and Estabrook, 1967a). This electron transport pathway involves the sequential interaction of NADPH, a flavoprotein (adrenodoxin reductase), a nonheme iron protein (adrenodoxin), and a hemoprotein (cytochrome P-450). Cytochrome P-450 is the terminal oxidase of this electron transport pathway and is responsible for both oxygen activation and substrate binding (Omura *et al.*, 1966; Wilson *et al.*, 1965; Imai and Sato, 1967a; Schenkman *et al.*, 1967; Cammer and Estabrook, 1967b). In addition, this hemoprotein is capable of binding carbon monoxide to form a complex exhibiting an absorption maximum at 450 mμ (Harding *et al.*, 1964; Omura *et al.*, 1965; Cooper *et al.*, 1965a). The spectral and enzymatic properties of this cytochrome are related to the hydrophobic nature of the hemo-

* From the Department of Biochemistry, Faculty of Medicine, University of British Columbia, Vancouver 8, B. C., Canada. *Received July 16, 1968.* We are grateful to the Medical Research Council of Canada and the National Cancer Institute of Canada for financial support.

protein which is closely associated with lipid. Any alteration of the hydrophobic nature of cytochrome P-450 results in its decomposition to cytochrome P-420, with an associated loss of hydroxylase activity. Such a conversion can be produced by a variety of agents including deoxycholate, urea, phospholipase, sulfhydryl reagents, and organic solvents (Omura and Sato, 1964a,b; Mason *et al.*, 1965; Cooper *et al.*, 1965b; Imai and Sato, 1967b; Ichikawa and Yamano, 1967).



DICUMAROL

In a previous communication from this laboratory (Williamson and O'Donnell, 1967) inhibition of NADPH-supported 11β-hydroxylation by dicumarol, a compound known to uncouple oxidative phosphorylation (Martius and Nitz-Litzow, 1953; Chance *et al.*, 1963), was reported. The inhibition of 11β-hydroxylation by dicumarol is distinct from its ability to uncouple oxidative phosphorylation because 11β-hydroxylation supported by NADPH does not require a high-energy intermediate such as ATP (Harding *et al.*, 1965; Guerra *et al.*, 1966). Therefore dicumarol must interact with a component of the 11β-hydroxylase pathway that results in inhibition of hydroxylation. The present communication discusses the interaction of dicumarol with cytochrome P-450, and the relationship between this interaction and the inhibition of steroid 11β-hydroxylation.

Materials and Methods

Preparation of 11β-Hydroxylase. The preparation of the beef adrenal mitochondrial acetone powder has been described previously (Williamson and O'Donnell, 1967). The 11β-hydroxylase system was extracted from the mitochondrial acetone powder immediately before use in incubations by the following procedure. Acetone powder (1 g) was homogenized in 30 ml of a solution of 0.154 M KCl (4°) and the homogenate after standing for 30 min at 4° was centrifuged at 17,000*g* for 30 min. The supernatant contained the 11β-hydroxylase system.

Incubations

General Conditions. The preparation and purification of solvents and steroids employed in these studies have been previously described (Williamson and O'Donnell, 1969a). Reaction mixtures were prepared and incubations were carried out as described previously (Williamson and O'Donnell, 1969a).

A solution (0.01 M) of dicumarol[1] was prepared by suspending 25 mg of the compound in 2.5 ml of 75% ethanol. A solution of 1 N KOH was added dropwise (4 or 5 drops) to the continually warmed suspension until all the dicumarol dissolved. The solution was then diluted to 7.5 ml with distilled water. An identical ethanol–water–1 N KOH solution not containing dicumarol was prepared and added in an equivalent quantity to each control reaction flask.

Inhibition of 11β-Hydroxylation as a Function of Dicumarol Concentration. Each reaction flask contained: 0.094 μmole of [1,2-^{3}H]deoxycorticosterone (specific activity 1.05 × 10^7 cpm/μmole) dissolved in 0.05 ml of propylene glycol, 1.35 ml of Tris-$MgCl_2$ buffer, 2.6 ml of enzyme preparation, 0.10 ml of dicumarol solution, nd NADPH (3.3 μmoles) dissolved in 0.2 ml of Trnis-$MgCl_2$ buffer. After 20-min incubation each reaction was terminated by the addition of ethyl acetate (5 ml).

Kinetic Studies on Dicumarol Inhibition of 11β-Hydroxylation. A typical incubation mixture contained: [1,2-^{3}H]deoxycorticosterone dissolved in 0.05 ml of propylene glycol, 0.70 ml of Tris-$MgCl_2$ buffer, 1.3 ml of enzyme preparation, and dicumarol dissolved in 0.05 ml of the ethanol–water–KOH solution. The mixture was kept in an ice–water bath throughout the additions. Each component was added at identical time intervals to each reaction flask. After 8-min incubation at 37°, NADPH (1.7 μmoles) dissolved in 0.05 ml of Tris-$MgCl_2$ buffer was added. After a further 90-sec incubation, a 1.0-ml portion was removed from each flask, pipetted into 2 ml of ice-cold ethyl acetate, and thoroughly mixed to terminate the reaction.

Isolation, Purification, and Measurement of Corticosterone. In the studies on the inhibition of 11β-hydroxylation as a function of dicumarol concentration, each ethyl acetate arrested reaction mixture was diluted with 10 ml of water, transferred to a separatory funnel, and extracted with ethyl acetate (four 15-ml portions). The ethyl acetate fraction was evaporated to dryness under reduced pressure.

In the kinetic experiments extractions were carried out in 12-ml centrifuge tubes. Each reaction sample was extracted with ethyl acetate (four 2-ml portions) and the ethyl acetate fraction was evaporated to dryness under nitrogen. Corticosterone was isolated from the ethyl acetate fractions, purified, and measured employing methods described previously (Williamson and O'Donnell, 1969a).

Effects of Deoxycorticosterone, Corticosterone, and Dicumarol on Cytochromes P-450 and P-420. Cytochromes P-450 and P-420 were measured under experimental conditions similar to those employed in the kinetic experiments. The experimental conditions and the methods employed to measure cytochromes P-450 and P-420 have been described previously (Williamson and O'Donnell, 1969a). Dicumarol was added to incubation mixtures dissolved in 0.15 ml of the ethanol–water–KOH solution.

Results

Kinetic Studies on Dicumarol Inhibition of Steroid 11β-Hydroxylation. The inhibition of 11β-hydroxylation

[1] Trivial names used: androstenedione, androst-4-ene-3,17-dione; adrenosterone, androst-4-ene-3,11,17-trione; deoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; corticosterone, 11β,21-dihydroxypregn-4-ene-3,20-dione; dicumarol, 3,3′-methylenebis(4-hydroxycoumarin).

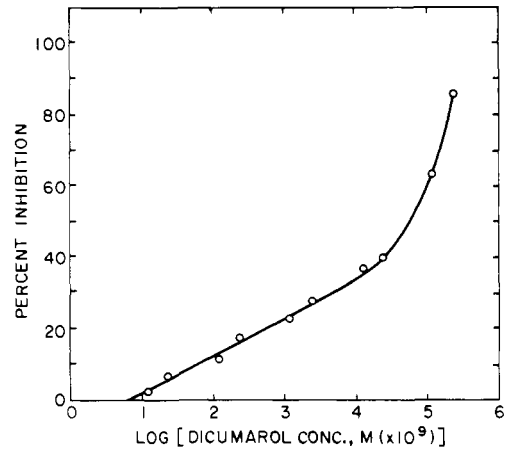


FIGURE 1: Inhibition of the 11β-hydroxylation of deoxycorticosterone as a function of dicumarol concentration. For reaction conditions, see Materials and Methods.

as a function of dicumarol concentration is shown in Figure 1. The reaction is inhibited by dicumarol at a concentration as low as 11.6 mμmoles/l. and inhibition reaches 36% at a dicumarol concentration of 11.6 μmoles/l. A further increase in dicumarol concentration results in a pronounced increase in the degree of inhibition of 11β-hydroxylation, 85% inhibition being obtained at a dicumarol concentration of 233 μmoles/l. The data suggest that dicumarol exerts more than one inhibitory action on the 11β-hydroxylation of deoxycorticosterone.

Kinetic data on dicumarol inhibition of 11β-hydroxylation are illustrated in Figures 2 and 3. In the plot of [S]/v *vs.* [S] shown in Figure 2, dicumarol, at the lowest concentration (58 μmoles/l.) examined, is a noncompetitive inhibitor of 11β-hydroxylation. However, at dicumarol concentrations of 145 and 233 μmoles per l., the inhibition is that of a mixed type, indicating that dicumarol has more than one mode of action on 11β-hydroxylation.

The complex nature of dicumarol inhibition of 11β-hydroxylation is readily demonstrated in the Dixon plot of 1/v *vs.* i (Dixon, 1953) shown in Figure 3. At substrate deoxycorticosterone concentrations of 7 and 35 μmoles per l. the kinetic curves are biphasic. At dicumarol concentrations less than 80 μmoles/l., the 11β-hydroxylase activity is relatively insensitive to changes in inhibitor concentrations as judged by the gradients of the lines joining the experimental values. At dicumarol concentrations greater than 80 μmoles/l. there is a distinct increase in the slopes of the kinetic curves. This additional inhibitory action of dicumarol is sensitive to substrate concentration. At a substrate deoxycorticosterone concentration of 35 μmoles/l., the slope change in the kinetic curve occurs at a higher dicumarol concentration than that observed with a deoxycorticosterone concentration of 7 μmoles/l. The degree of the slope change is also greatly diminished suggesting that under these conditions inhibition may be competitive with substrate.

Effects of Deoxycorticosterone and Dicumarol on Cytochromes P-450 and P-420. The role of cytochrome P-450 as both the oxygen-activating (Estabrook *et al.*, 1963; Wilson *et al.*, 1965) and substrate-binding (Schenkman *et al.*, 1967; Imai and Sato, 1967a) component of mixed-function oxidases suggests that dicumarol inhibition of 11β-hydroxylation may result from interaction of this compound with cytochrome P-450. Therefore, the effects of dicumarol and substrate deoxycortiscosterone on cytochrome P-450 were investigated under experimental conditions similar to those employed in the kinetic experiments.

Preliminary experiments revealed that cytochrome P-450, while stable during incubation at temperatures

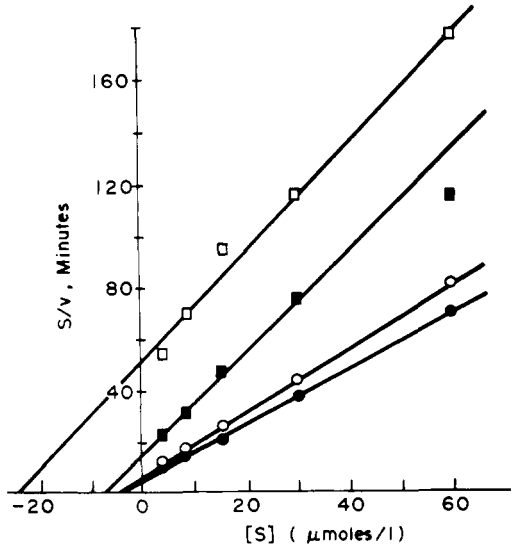


FIGURE 2: [S]/v *vs.* [S] plot of dicumarol inhibition of the 11β-hydroxylation of deoxycorticosterone. For reaction conditions, see Materials and Methods. The specific activity of the substrate [1,2-^{3}H]deoxycorticosterone was 6.24 × 10^7 cpm/μmole. Final concentration (micromoles per liter) of dicumarol: 0 (●), 58 (○), 145 (■), and 233 (□).

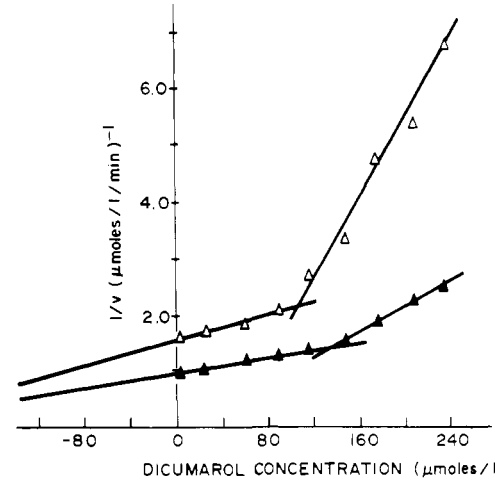


FIGURE 3: 1/v *vs.* i plot of dicumarol inhibition of 11β-hydroxylation of deoxycorticosterone. For reaction conditions, see Materials and Methods. The specific activity of the substrate [1,2-^{3}H]deoxycorticosterone was 4.04 × 10^7 cpm/μmole. Final concentration (micromoles per liter) of [1,2-^{3}H]deoxycorticosterone: 7 (△) and 35 (▲).

below 30°, undergoes spontaneous decomposition to cytochrome P-420 during incubation at 37 or 45°. However, if incubations are carried out in the presence of substrate deoxycorticosterone, then the recovery of cytochrome P-450 is increased (Figure 4). The increased recovery of cytochrome P-450 is observed at concentrations of deoxycorticosterone in the region of its K_m (5.5 μmoles/l.) for 11β-hydroxylation. Incubations carried out in the presence of corticosterone, the 11β-hydroxylated product of deoxycorticosterone, do not result in increased recoveries of cytochrome P-450 (Figure 4).

Sharma *et al.* (1963) have observed that androstenedione is a competitive inhibitor of the 11β-hydroxylation of deoxycorticosterone. In the present studies it was demonstrated that androstenedione, like deoxycorticosterone, increases the recovery of cytochrome P-450. However, the presence of an 11-oxo group in adrenosterone completely eliminates this effect. Similar studies on the effect of the 11β-hydroxylase inhibitor dicumarol on the recovery of cytochrome P-450 were carried out. Dicumarol at the lowest concentration, 23 μmoles/l., produces an increase in the recovery of cytochrome P-450 (Figure 5). Dicumarol concentrations lower than those shown in Figure 5 have been examined, and significant increases in cytochrome P-450 are observed with dicumarol at concentrations as low as 2.3 μmoles/l. Therefore dicumarol increases the recovery of cytochrome P-450 at concentrations at which it is a noncompetitive inhibitor of 11β-hydroxylation. At dicumarol concentrations greater than 23 μmoles/l., the recovery of cytochrome P-450 decreases, and cytochrome P-420 correspondingly increases (Figure 5). At a dicumarol concentration of 145 μmoles/l., all the cytochrome P-450 originally present in the enzyme preparation has disappeared.

The effect of substrate deoxycorticosterone on the recovery of cytochrome P-450 as a function of dicumarol concentration is shown in Figure 6. In the absence of dicumarol, the addition of deoxycorticosterone (7 or 34 μmoles per l.) produces an increase in cytochrome P-450. The increased recovery of cytochrome P-450 in the presence of deoxycorticosterone has been demonstrated previously (Figure 4) and at a concentration of 34 μmoles/l., the deoxycorticosterone effect is maximal. The subsequent addition of dicumarol (23 μmoles/l.) produces a further increase in cytochrome P-450 (Figure 6). The extent of this increase in cytochrome P-450 is essentially the same in the presence of deoxycorticosterone (7 or 34 μmoles per l.) as in its absence, indicating that the effects of dicumarol and deoxycorticosterone on cytochrome P-450 are independent and additive. As the dicumarol concentration is increased above 60 μmoles/l., there is a decrease in cytochrome P-450 recovery at both steroid concentrations (Figure 6). However the dicumarol concentration at which the decrease in cytochrome P-450 is first observed is higher in the presence of substrate than in its absence. The general profile of the loss of cytochrome P-450 is the same in both the presence and absence of deoxycorticosterone, but at any given

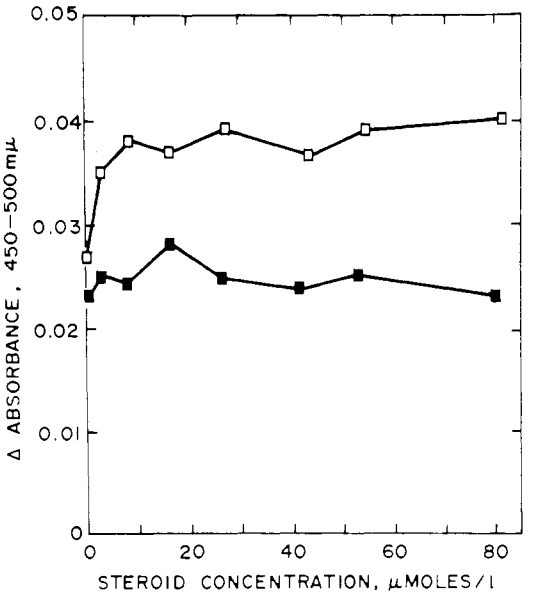


FIGURE 4: Recovery of cytochrome P-450 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of deoxycorticosterone or corticosterone. For reaction conditions, see Materials and Methods. After 8-min incubation NADPH (3.3 μmoles) dissolved in 0.15 ml of Tris-$MgCl_2$ buffer was added to each flask and the incubation continued for a further 90 sec. Steroid added: deoxycorticosterone (□), corticosterone (■).

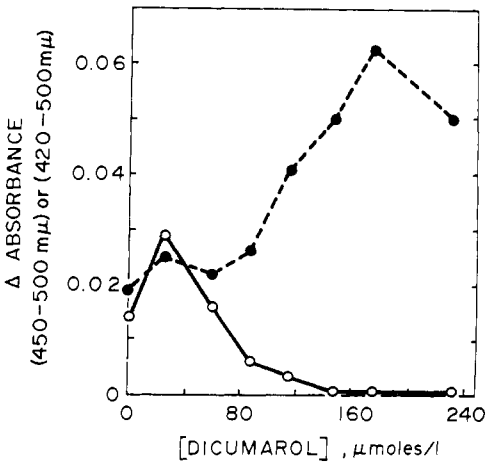


FIGURE 5: Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of dicumarol. For reaction conditions, see Materials and Methods. NADPH (4.9 μmoles) dissolved in 0.15 ml of Tris-$MgCl_2$ buffer was added after 8-min incubation and the incubation continued for a further 90 sec. Cytochrome P-450 (○), cytochrome P-420 (●).

dicumarol concentration the presence of deoxycorticosterone increases the amount of cytochrome P-450 recovered.

The effect of dicumarol on the rate of disappearance of cytochrome P-450 during incubation at 37° is shown in Figure 7. Incubations were carried out at two dicumarol concentrations: one concentration (23 μmoles/l.) was in the region of noncompetitive inhibition of 11β-hydroxylation (Figures 2 and 3) while the other (233 μmoles/l.) was in the region of mixed

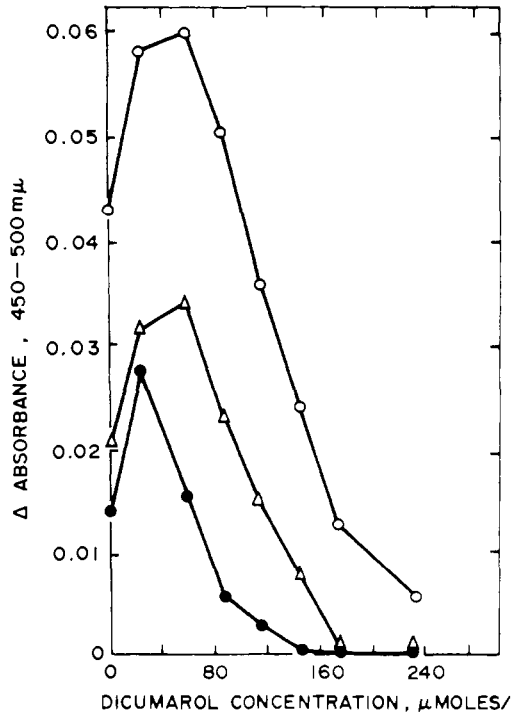


FIGURE 6: Recovery of cytochrome P-450 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of deoxycorticosterone and dicumarol. For incubation conditions, see Materials and Methods and Figure 5. Deoxycorticosterone final concentration (micromoles per liter): 0 (●), 7.4 (△), and 34 (○).

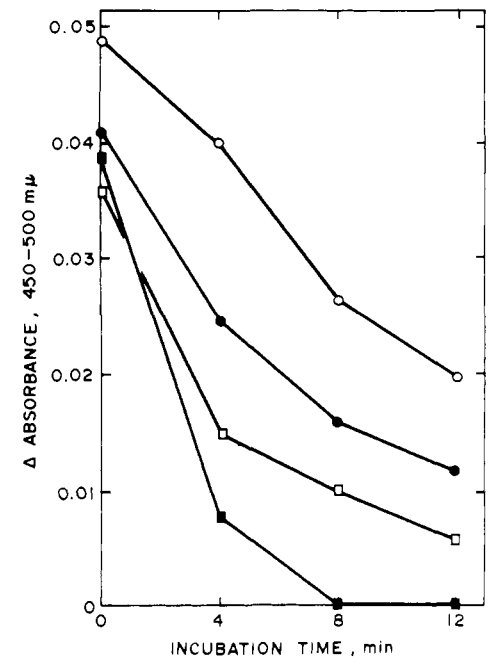


FIGURE 7: Effect of deoxycorticosterone and dicumarol on the rate of cytochrome P-450 disappearance. For incubation conditions, see Materials and Methods. Control (□); plus deoxycorticosterone, final concentration 39 μmoles/l. (○); plus dicumarol, final concentration 23 (●) or 233 (■) μmoles/l.

inhibition of 11β-hydroxylation. At the lower concentration dicumarol diminishes the rate of decomposition of cytochrome P-450 (Figure 7). The effect of dicumarol at this concentration is analogous to that observed with substrate deoxycorticosterone (Figure 7) but the decrease in cytochrome P-450 disappearnace is not as pronounced as that observed with deoxycorticosterone. At a concentration of 233 μmoles/l. dicumarol enhances the rate of decomposition of cytochrome P-450, an effect opposite to that observed at the lower dicumarol concentration.

Effect of Dicumarol on the Substrate-Induced Difference Spectrum of Cytochrome P-450. Recent studies have revealed that the addition of substrates to mitochondrial and microsomal hydroxylase systems produce spectral changes that can be measured by the technique of difference spectrophotometry (Cooper *et al.*, 1965a; Schenkman *et al.*, 1967; Imai and Sato, 1967a; Cammer and Estabrook, 1967b). Evidence indicates that the spectral changes result from interaction of substrates with cytochrome P-450 (Imai and Sato, 1967a). In an accompanying paper (Williamson and O'Donnell, 1969b) it was demonstrated that Metopirone, a competitive inhibitor of 11β-hydroxylation, inhibits the difference spectrum produced by interaction of cytochrome P-450 with deoxycorticosterone. Similar experiments were carried out with the inhibitor dicumarol. Interaction of dicumarol with cytochrome P-450 could not be examined spectrophotometrically because dicumarol itself absorbs in the region of the spectrum employed to measure these interactions. However, the effect of dicumarol on the substrate deoxycorticosterone-induced difference spectrum was examined. Dicumarol, at low concentrations which inhibit 11β-hydroxylation noncompetitively, does not affect the difference spectrum induced in cytochrome P-450 by deoxycorticosterone. Thus low concentrations of dicumarol do not prevent the interaction of deoxycorticosterone with cytochrome P-450. However at high concentrations of dicumarol the deoxycorticosterone-induced difference spectrum is diminished because of the destruction of cytochrome P-450 by these concentrations of inhibitor. This effect of dicumarol on the substrate-induced difference spectrum is diminished by an increase in substrate deoxycorticosterone concentration.

Discussion

The ability of substrates of both mitochondrial and microsomal hydroxylase systems to produce spectral changes in cytochrome P-450 indicates that this hemoprotein is the substrate-binding component (Schenkman *et al.*, 1967; Imai and Sato, 1967a; Cammer and Estabrook, 1967b). The precise mechanism whereby these substrates induce such spectral changes in cytochrome P-450 is not known. Imai and Sato (1967a) and Schenkman *et al.* (1967) suggest that the binding of substrate to cytochrome P-450 causes a conformational alteration with an accompanying modification of ligand interaction with the heme iron. However, Oldham *et al.* (1968) suggest that the spectral change results from

oxidation of a moiety in cytochrome P-450 on addition of substrate. The present communication shows that in addition to the spectral changes in cytochrome P-450 produced by deoxycorticosterone, the interaction of the steroid with cytochrome P-450 results in stabilization of the hemoprotein. Thus if the spectral changes produced by cytochrome P-450 by interaction with deoxycorticosterone reflect a conformational change in the hemoprotein, then the altered conformation is more resistant to degradation. Alternatively, the interaction of deoxycorticosterone with cytochrome P-450 may protect a labile group in the hemoprotein, hence preventing its decomposition. A similar stabilization of cytochrome P-450 is observed with the substrate androstenedione. However, cytochrome P-450 recoveries are not influenced in incubations with corticosterone or adrenosterone, indicating that there is no interaction between cytochrome P-450 and the 11-oxygenated steroids. Thus the stabilization of cytochrome P-450 is specific for steroid substrates.

The inhibition of 11β-hydroxylation by dicumarol results from its effects on cytochrome P-450. Low concentrations of the inhibitor produce an increased recovery of cytochrome P-450, indicating that the inhibitor binds to the cytochrome and increases its stability in a manner similar to deoxycorticosterone. The protective effects of deoxycorticosterone and dicumarol on cytochrome P-450 are additive. Moreover dicumarol, at concentrations that stabilize cytochrome P-450, is a noncompetitive inhibitor of 11β-hydroxylation. These results suggest that dicumarol and deoxycorticosterone bind to different sites on the cytochrome and that the binding of one compound does not interfere with the binding of the other. This is supported by the finding that low concentrations of dicumarol do not affect the spectral changes produced by the interaction of deoxycorticosterone with cytochrome P-450. However, the binding of dicumarol to cytochrome P-450 does result in an inhibition of 11β-hydroxylation, perhaps by interfering with the interaction of steroid substrate and oxygen on the hemoprotein.

At high concentrations dicumarol increases the rate of decomposition and hence the instability of cytochrome P-450, an effect opposite to that observed at low dicumarol concentrations. A similar conversion of cytochrome P-450 into cytochrome P-420 is observed with a wide variety of agents (Omura and Sato, 1964b; Imai and Sato, 1967b; Mason *et al.*, 1965; Cooper *et al.*, 1965b; Ichikawa and Yamano, 1967). The decomposition of cytochrome P-450 results from alteration of the hydrophobic environment of the hemoprotein which is essential for the maintenance of its unique enzymatic and spectral properties. The inhibition of 11β-hydroxylation produced by high concentrations of dicumarol most likely results from the enhanced conversion of cytochrome P-450 into cytochrome P-420.

The effects of high concentrations of dicumarol on both 11β-hydroxylation and cytochrome P-450 are sensitive to steroid substrate. This inhibitory action of dicumarol on 11β-hydroxylation is greatly diminished by an increase in deoxycorticosterone concentration. Cytochrome P-450 recoveries at any given dicumarol concentration are increased in the presence of deoxycorticosterone. Thus at high dicumarol concentrations there is competition between deoxycorticosterone and dicumarol for binding to cytochrome P-450.

The structural features of dicumarol that permit interaction with cytochrome P-450 resulting in inhibition of 11β-hydroxylation are not known. Further elucidation of the mechanism of dicumarol inhibition of 11β-hydroxylation must await an understanding of the complex nature of cytochrome P-450 and of the characteristics of the substrate and inhibitor binding sites.

Acknowledgments

We thank the Wisconsin Agricultural Research Foundation and Abbott Laboratories, North Chicago, Ill., for gifts of dicumarol. The beef adrenal glands were kindly provided by Canada Packers Limited, Vancouver, B. C.

References

Cammer, W., and Estabrook, R. W. (1967a), *Arch. Biochem. Biophys. 122*, 721.

Cammer, W., and Estabrook, R. W. (1967b), *Arch. Biochem. Biophys. 122*, 735.

Chance, B., Williams, G. R., and Hollunger, G. (1963), *J. Biol. Chem. 238*, 439.

Cooper, D. Y., Narasimhulu, S., Slade, A., Foroff, O., and Rosenthal, O. (1965a), *Life Sci. 4*, 2109.

Cooper, D. Y., Narasimhulu, S., Rosenthal, O., and Estabrook, R. W. (1965b), *in* Oxidase and Related Redox Systems, King, T., Morrison, M., and Mason, H. S., Ed., New York, N. Y., Wiley, p 838.

Dixon, M. (1953), *Biochem. J. 55*, 170.

Estabrook, R. W., Cooper, D. Y., and Rosenthal, O. (1963), *Biochem. Z. 338*, 741.

Guerra, F., Péron, F. G., and McCarthy, J. L. (1966), *Biochim. Biophys. Acta 117*, 433.

Harding, B. W., and Nelson, D. (1966), *J. Biol. Chem. 241*, 2212.

Harding, B. W., Wilson, L. D., Wong, S. H., and Nelson, D. (1965), *Steroids Suppl. II*, 51.

Harding, B. W., Wong, S. H., and Nelson, D. H. (1964), *Biochim. Biophys. Acta 92*, 415.

Ichikawa, Y., and Yamano, T. (1967), *Biochim. Biophys. Acta 147*, 518.

Imai, Y., and Sato, R. (1967a), *J. Biochem. (Tokyo) 62*, 239.

Imai, Y., and Sato, R. (1967b), *European J. Biochem. 1*, 419.

Martius, C., and Nitz-Litzow, D. (1953), *Biochim. Biophys. Acta 12*, 134.

Mason, H. S., North, J. C., and Vanneste, M. (1965), *Federation Proc. 24*, 1172.

Nakamura, Y., and Otsuka, H. (1966), *Biochim. Biophys. Acta 122*, 34.

Oldham, S. B., Wilson, L. D., Landgraf, W. L., and Harding, B. W. (1968), *Arch Biochem. Biophys. 123*, 484.

Omura, T., Sanders, E., Estabrook, R. W., Cooper, D. Y., and Rosenthal, O. (1966), *Arch. Biochem. Biophys. 117*, 660.
Omura, T., and Sato, R. (1964a), *J. Biol. Chem. 239*, 2370.
Omura, T., and Sato, R. (1964b), *J. Biol. Chem. 239*, 2379.
Omura, T., Sato, R., Cooper, D. Y., Rosenthal, O., and Estabrook, R. W. (1965), *Federation Proc. 24*, 1181.
Schenkman, J. B., Remmer, H., and Estabrook, R. W. (1967), *Mol. Pharmacol. 3*, 113.
Sharma, D. C., Forchielli, E., and Dorfman, R. I. (1963), *J. Biol. Chem. 238*, 572.
Williamson, D. G., and O'Donnell, V. J. (1967), *Can. J. Biochem. 45*, 340.
Williamson, D. G., and O'Donnell, V. J. (1969a), *Biochemistry 8*, 1289 (this issue; paper I).
Williamson, D. G., and O'Donnell, V. J. (1969b), *Biochemistry 8*, 1306 (this issue; paper III).
Wilson, L. D., Nelson, D. H., and Harding, B. W. (1965), *Biochim. Biophys. Acta 99*, 391.

The Interaction of Metopirone with Adrenal Mitochondrial Cytochrome P-450. A Mechanism for the Inhibition of Adrenal Steroid 11β-Hydroxylation*

Denis G. Williamson and Vincent J. O'Donnell

ABSTRACT: The mechanism of Metopirone inhibition of the 11β-hydroxylation of deoxycorticosterone has been studied with an 11β-hydroxylase extracted from an acetone powder of beef adrenal mitochondria. Kinetic data indicate that the inhibition is competitive with substrate deoxycorticosterone, the K_i of Metopirone being 1.0×10^{-7} mole/l. Metopirone inhibition of 11β-hydroxylation results from the interaction of this compound with cytochrome P-450, the oxygen-activating and steroid-binding component of the 11β-hydroxylase system. The decomposition of cytochrome P-450 during incubation of the enzyme preparation at 37° is prevented by Metopirone at concentrations that also inhibit 11β-hydroxylation. A similar stabilization of cytochrome P-450 is exerted by substrate deoxycorticosterone. These results indicate that both Metopirone and deoxycorticosterone bind to cytochrome P-450, preventing its degradation. However, the effects of Metopirone and deoxycorticosterone on cytochrome P-450 are not additive, the recovery of the hemoprotein being no greater after incubation with both Metopirone and deoxycorticosterone than with Metopirone alone. Therefore the two compounds are competing for the same binding site on cytochrome P-450. Moreover, Metopirone, at concentrations that inhibit 11β-hydroxylation, also inhibits the spectral changes produced by interaction of deoxycorticosterone with cytochrome P-450. This inhibition can be substantially overcome by an increase in deoxycorticosterone concentration, confirming the competition between Metopirone and deoxycorticosterone for a binding site on cytochrome P-450. Metopirone itself, at these concentrations, does not induce detectable spectral changes in cytochrome P-450. It is the ability of Metopirone to interfere with the binding of substrate deoxycorticosterone to cytochrome P-450 that results in an inhibition of the 11β-hydroxylation reaction.

Metopirone [2-methyl-1,2-bis(3-pyridyl)-1-propanone, SU-4885] inhibition of corticosteroid biogenesis is well documented (Chart *et al.*, 1958; Liddle *et al.*, 1958; Dominguez and Samuels, 1963; Sharma *et al.*, 1963; Ertel and Ungar, 1964; Levy *et al.*, 1965). While this compound was first thought to be a specific inhibitor of steroid 11β-hydroxylation, inhibition of hydroxylations at the C-18 (Kraulis and Birmingham, 1965; Sanzari and Péron, 1966) and C-19 (Griffiths, 1963) positions have also been reported. The inhibition of Metopirone appears to be associated with hydroxylations of the steroid that occur in the mitochondrion.

The mechanism of Metopirone inhibition of steroid hydroxylations is unclear. Dominguez and Samuels (1963) demonstrated that Metopirone is a competitive inhibitor of steroid 11β-hydroxylation by rat adrenal homogenates. A similar result was obtained in our laboratory on studies with Metopirone inhibition of the 11β-hydroxylation of deoxycorticosterone with an enzyme preparation extracted from an acetone powder of beef adrenal mitochondria (Williamson and O'Donnell, 1967).

While direct competition between steroid substrates and Metopirone for the hydroxylase is established, Sanzari and Péron (1966) suggest that Metopirone may also interact with other factors involved in steroid

* From the Department of Biochemistry, Faculty of Medicine, University of British Columbia, Vancouver 8, B. C., Canada. *Received July 19, 1968.* We are grateful to the Medical Research Council of Canada and the National Cancer Institute of Canada for financial support.