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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

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hydroxylation reactions. They reported that in 11β-hydroxylation supported by isocitrate, but not by succinate, Metopirone inhibition may also be mediated by its action on intramitochondrial NADPH formation. However, Dominguez and Samuels (1963) have observed that Metopirone has no effect on NADPH formation by a regenerating system consisting of NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase. Moreover, Ertel and Ungar (1964) reported that Metopirone inhibition is not reversed by addition of NADPH.

Recently Harding *et al.* (1966) and Wilson *et al.* (1967) have presented preliminary evidence that Metopirone may interfere with the interaction of steroid substrates and cytochrome P-450, the oxygen-activating and steroid-binding component of the 11β-hydroxylase (Estabrook *et al.*, 1963; Wilson *et al.*, 1965; Omura *et al.*, 1965, Cooper *et al.*, 1965; Imai and Sato, 1967; Schenkman *et al.*, 1967; Cammer and Estabrook, 1967). The present communication describes detailed studies on the interaction of Metopirone with the 11β-hydroxylase obtained by extraction of an acetone powder of beef adrenal mitochondria. In particular, the interaction of Metopirone and cytochrome P-450 was examined, and is causally related to the inhibition of the 11β-hydroxylation of deoxycorticosterone by Metopirone.

Materials and Methods

Chemicals. The preparation and purification of solvents, chemicals, and steroids employed in these studies have been previously described (Williamson and O'Donnell, 1969a). Metopirone[1] was a gift from Ciba Company Limited.

Incubation Conditions. The preparation of the beef adrenal mitochondrial acetone powder and extraction of the 11β-hydroxylase from the acetone powder with a 0.154 M KCl solution have been described (Williamson and O'Donnell, 1967). The incubation conditions and methods described in the preceding paper (Williamson and O'Donnell, 1969b) were employed for the kinetic studies on Metopirone inhibition, for studies on the effects of Metopirone on cytochrome P-450, and for the isolation and radioassay of corticosterone. These procedures have been described in detail elsewhere (Williamson and O'Donnell, 1967, 1969a).

The fraction of the enzyme preparation precipitating between 20 and 40% saturation with ammonium sulfate was employed for the studies on spectral changes produced by the interaction of deoxycorticosterone with cytochrome P-450. The 20–40% ammonium sulfate precipitate obtained from 8 ml of the enzyme preparation was dissolved in 1.6 ml of 0.0375 M Tris buffer (pH 7.4) containing 9.4×10^{-4} M $MgCl_2$ and 0.096 M KCl. The mixture was divided equally between two cuvets and a base line from 500 to 375 mμ was recorded. Deoxycorticosterone dissolved in 0.02 ml of propylene glycol was added to the sample cuvet. An identical quantity of propylene glycol was added to the reference cuvet. The difference spectrum was then recorded at room temperature. Metopirone dissolved in 0.01 ml of Tris-$MgCl_2$ buffer was then added to both the sample and reference cuvets and the difference spectrum was again recorded. A control experiment in which 0.01 ml of Tris-$MgCl_2$ buffer alone was added to each cuvet was carried out. This addition was found to have no detectable effect on the magnitude of the deoxycorticosterone-induced difference spectrum. For each difference spectrum obtained, the base line was subtracted from the change in light absorbance produced by addition of deoxycorticosterone and Metopirone, and the calculated difference spectrum was plotted.

The experimental conditions employed for measurement of the Metopirone-induced spectral changes in cytochrome P-450 were the same as those used to measure the deoxycorticosterone-induced changes. Propylene glycol (0.02 ml) was added to both the sample and reference cuvets. After recording the base line, Metopirone dissolved in 0.01 ml of Tris-$MgCl_2$ buffer was added to the sample cuvet. An identical quantity of Tris-$MgCl_2$ buffer was added to the reference cuvet. The difference spectrum was then recorded.

Results

Kinetic Studies on Metopirone Inhibition of 11β-Hydroxylation. Previous investigation of the inhibition of the 11β-hydroxylase by Metopirone indicated that this compound effectively inhibits 11β-hydroxylation at concentrations much lower than that of the substrate deoxycorticosterone (Williamson and O'Donnell, 1967). The results of kinetic studies on Metopirone inhibition of the 11β-hydroxylation reaction are shown in Figure 1. In the Dixon plot of $1/v$ *vs.* i, the straight lines joining the experimental values obtained at each

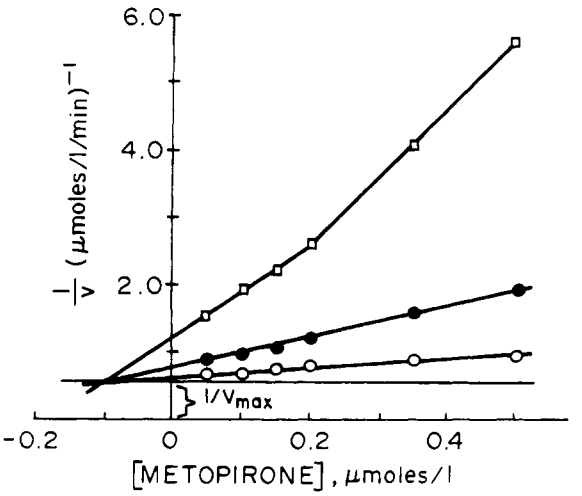

FIGURE 1: $1/v$ *vs.* i plot of Metopirone inhibition of the 11β-hydroxylation of deoxycorticosterone. For reaction conditions, see Materials and Methods. Final concentration (micromoles per liter) of [1,2-^{3}H]deoxycorticosterone (specific activity 7.2×10^7 cpm/μmole): 3.35 (□), 10.2 (●), and 30.5 (○).

[1] Trivial names used: deoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; corticosterone, 11β,21-dihydroxypregn-4-ene-3,20-dione; deoxycortisol, 17α,21-dihydroxypregn-4-ene-3,20-dione; Metopirone, SU-4885, 2-methyl-1,2-bis(3-pyridyl)-1-propanone; dicumarol, 3,3′-methylenebis(4-hydroxycoumarin).

substrate concentration intersect at a point above the abcissa corresponding to $1/V_{max}$ determined in the absence of inhibitor. This indicates that Metopirone is a competitive inhibitor of the 11β-hydroxylation of deoxycorticosterone (Dixon, 1953). The point of intersection also gives the inhibitor constant, K_i, for Metopirone of 1.0×10^{-7} mole/l. This value is much lower than the K_m of 5.5×10^{-6} mole/l. for the 11β-hydroxylation of deoxycorticosterone. The kinetic curve obtained with the lowest substrate deoxycorticosterone concentration, 3.35 μmoles/l. (Figure 1), is biphasic, the slope increasing as the Metopirone concentration is increased. This slope change is not observed with the higher substrate concentrations.

Effect of Metopirone on Cytochromes P-450 and P-420. Studies on the inhibition of 11β-hydroxylation by dicumarol (Williamson and O'Donnell, 1969b) demonstrated that both the inhibitor dicumarol and the substrate deoxycorticosterone affect cytochrome P-450. The hemoprotein extracted from an acetone powder of beef adrenal mitochondria is unstable, undergoing spontaneous decomposition to cytochrome P-420 during incubation at 37°. However, if the enzyme preparation is incubated in the presence of substrate deoxycorticosterone, then both the extent and rate of decomposition of cytochrome P-450 are diminished. Substrate deoxycorticosterone stabilizes the cytochrome, either by protecting a labile moiety or by transforming the hemoprotein to a more stable conformation. Similar experiments in the present communication were carried out with Metopirone to determine whether this compound has any effect on cytochrome P-450. Figure 2 shows the recovery of cytochromes P-450 and P-420 after incubation of the enzyme preparation with Metopirone at concentrations ranging from 1.7 to 220 μmoles per l. At low concentrations, Metopirone produces a large increase in the cytochrome P-450 recovered after 8-min incubation over

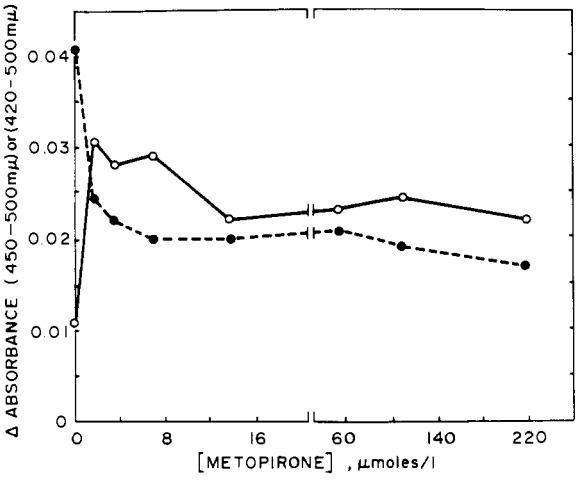

FIGURE 2: Recovery of cytochromes P-450 and P-420 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of metopirone. For reaction conditions, see Materials and Methods. Incubations were carried out for 8 min at 37°. Cytochrome P-450 (○), cytochrome P-420 (●).

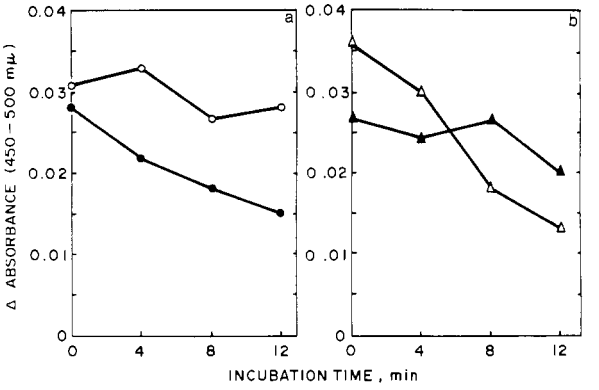

FIGURE 3: Effect of Metopirone on the rate of disappearance of cytochrome P-450. For reaction conditions, see Materials and Methods. Incubations were carried out at 37° for 0, 4, 8, and 12 min. (a) Control (●); plus Metopirone, 3.5 μmoles/l. final concentration (○). (b) Control (△); plus Metopirone, 27.2 μmoles/l. final concentration (▲).

that recovered in its absence. The effect of Metopirone at concentrations lower than those illustrated in Figure 2 has been investigated; a maximal increase in cytochrome P-450 is observed at a Metopirone concentration of 0.5 μmole/l. and a significant increase is evident at a concentration of 0.05 μmole/l. Therefore Metopirone increases the level of cytochrome P-450 at concentrations in the region of the K_i (0.1 μmole/l.) for 11β-hydroxylase inhibition. As the Metopirone concentration is increased, there is a slight decrease in the amount of recovered cytochrome P-450 to a level that is not influenced by further increases in Metopirone concentration. The levels of cytochrome P-420 decrease at low concentrations of Metopirone and, as the inhibitor concentration is increased, remain low and constant.

A time study on the rate of decomposition of cytochrome P-450 in the presence and absence of Metopirone was carried out. Two concentrations (3.5 and 27.2 μmoles/l.) of Metopirone were employed and the results are shown in Figure 3. At the former concentration, where the recovery of cytochrome P-450 is high (Figure 2), Metopirone almost eliminates the time-dependent loss of cytochrome P-450 observed in the absence of the inhibitor (Figure 3a). At the higher Metopirone concentration (27.2 μmoles/l.) less cytochrome P-450 is recovered at zero time in the presence of inhibitor than in its absence (Figure 3b). However, the rate of disappearance of cytochrome P-450 is greater in the absence of Metopirone than in its presence. Therefore as the incubation period progresses, the level of cytochrome P-450 in the absence of Metopirone is less than that observed in the presence of the inhibitor.

The interrelationship between the effects of deoxycorticosterone and Metopirone on cytochrome P-450 was ascertained by the addition of the two compounds to each reaction mixture. The effects of increasing Metopirone concentrations on cytochrome P-450 in both the absence and presence of deoxycorticosterone are shown in Figure 4. Both deoxycorticosterone and

low concentrations of Metopirone produce an increase in cytochrome P-450. These effects are demonstrated (Figure 4) either when deoxycorticosterone is added (no Metopirone) or when Metopirone is added (1 μmole/l., no deoxycorticosterone). However, addition of both deoxycorticosterone (42 μmoles/l.) and Metopirone (1 μmole/l.) does not produce an increase in cytochrome P-450 greater than that produced by Metopirone alone. Thus the effects of the two compounds on cytochrome P-450 are not additive. The recovery of cytochrome P-450 is greater in the presence of Metopirone (1 μmole/l.) alone than in the presence of deoxycorticosterone (42 μmoles/l.) alone, a finding that may reflect the higher affinity of the 11β-hydroxylase for Metopirone (K_i, 1.0×10^{-7} mole/l.) than for deoxycorticosterone (K_m, 5.5×10^{-6} mole/l.). As the Metopirone concentration is increased, the recovery of cytochrome P-450 decreases (Figure 4). However, deoxycorticosterone virtually eliminates the decrease in cytochrome P-450 observed at the higher Metopirone concentrations.

Metopirone-Induced Difference Spectrum of Cytochrome P-450. Schenkman *et al.* (1967) and Imai and Sato (1967) have demonstrated that substrates and inhibitors of mixed-function oxidases interact with cytochrome P-450 producing spectral changes in the hemoprotein. The difference spectrum induced by addition of Metopirone to a mitochondrial cytochrome P-450 preparation is shown in Figure 5A. The difference spectrum exhibits an absorption maximum at 422 mμ and a minimum at 400–405 mμ. Significant spectral changes are observed only at Metopirone concentrations of 20 μmoles/l. and greater. Thus the concentration of Metopirone required to induce a significant spectral change in cytochrome P-450 is higher than that required to inhibit 11β-hydroxylation (Figure 1).

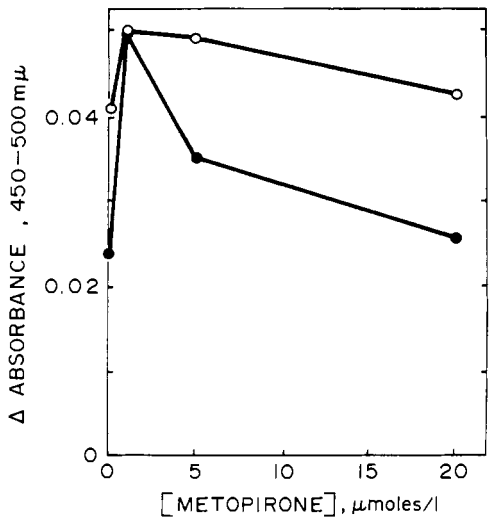

FIGURE 4: Recovery of cytochrome P-450 from the mitochondrial acetone powder enzyme preparation after incubation in the presence of Metopirone and deoxycorticosterone. For reaction conditions, see Materials and Methods. Incubations were carried out at 37° for 8 min. Metopirone (●); Metopirone plus deoxycorticosterone, 42 μmoles/l. final concentration (0).

Effect of Metopirone on the Substrate Deoxycorticosterone-Induced Difference Spectrum of Cytochrome P-450. It has been demonstrated that deoxycorticosterone induces spectral changes in cytochrome P-450 (D. G. Williamson and V. J. O'Donnell, unpublished data). The substrate concentration (7 μmoles/l.) that induces a half-maximal spectral change corresponds to the K_m (5.5 μmoles/l.) of deoxycorticosterone for 11β-hydroxylation. The deoxycorticosterone-induced difference spectrum and the effect of Metopirone on this difference spectrum are shown in Figure 5B, C. The spectrum exhibits a minimum at 420 mμ and a maximum at 385 mμ. The subsequent addition of Metopirone to the incubation mixture containing substrate deoxycorticosterone (2.4 μmoles/l.) results in a diminution of the difference spectrum. Metopirone (0.2 μmole/l.) inhibits the substrate-induced difference spectrum by more than 50% (Figure 5B). Therefore, Metopirone diminishes the substrate-induced difference spectrum at concentrations where inhibition of 11β-hydroxylation occurs (Figure 1). The inhibition of the deoxycorticosterone-induced difference spectrum is substantially overcome by an increase in steroid substrate concentration (Figure 5C).

Discussion

In the preceding paper (Williamson and O'Donnell, 1969b) it was demonstrated that the presence of substrate deoxycorticosterone in the incubation mixture diminishes both the extent and rate of degradation of cytochrome P-450. This hemoprotein is the oxygen-activating and steroid-binding component of mixed-function oxidases (Estabrook *et al.*, 1963; Wilson *et al.*, 1965; Imai and Sato, 1967; Schenkman *et al.*, 1967; Cammer and Estabrook, 1967). These findings suggest that the substrate is binding to the hemoprotein and thereby stabilizing it, either by altering the conformation of the cytochrome to a more stable form, or by protecting a labile moiety in the hemoprotein. Metopirone, at concentrations which effectively inhibit 11β-hydroxylation, stabilizes cytochrome P-450 in a manner analogous to substrate deoxycorticosterone. Therefore Metopirone inhibition of steroid 11β-hydroxylation appears to be associated with the ability of the inhibitor to interact with cytochrome P-450.

Although both deoxycorticosterone and Metopirone at low concentrations stabilize cytochrome P-450, the effects of these compounds are not additive indicating that the binding of one compound interferes with the binding of the other, resulting in competitive inhibition of deoxycorticosterone 11β-hydroxylation by Metopirone. In contrast, dicumarol at low concentrations is a noncompetitive inhibitor of the 11β-hydroxylation of deoxycorticosterone (Williamson and O'Donnell, 1969b). At these concentrations dicumarol also protects cytochrome P-450 in a manner analogous to that observed with deoxycorticosterone and Metopirone. However, the stabilizing effects of dicumarol and substrate deoxycorticosterone are additive, indicating that the two compounds are binding to different sites on the hemoprotein.

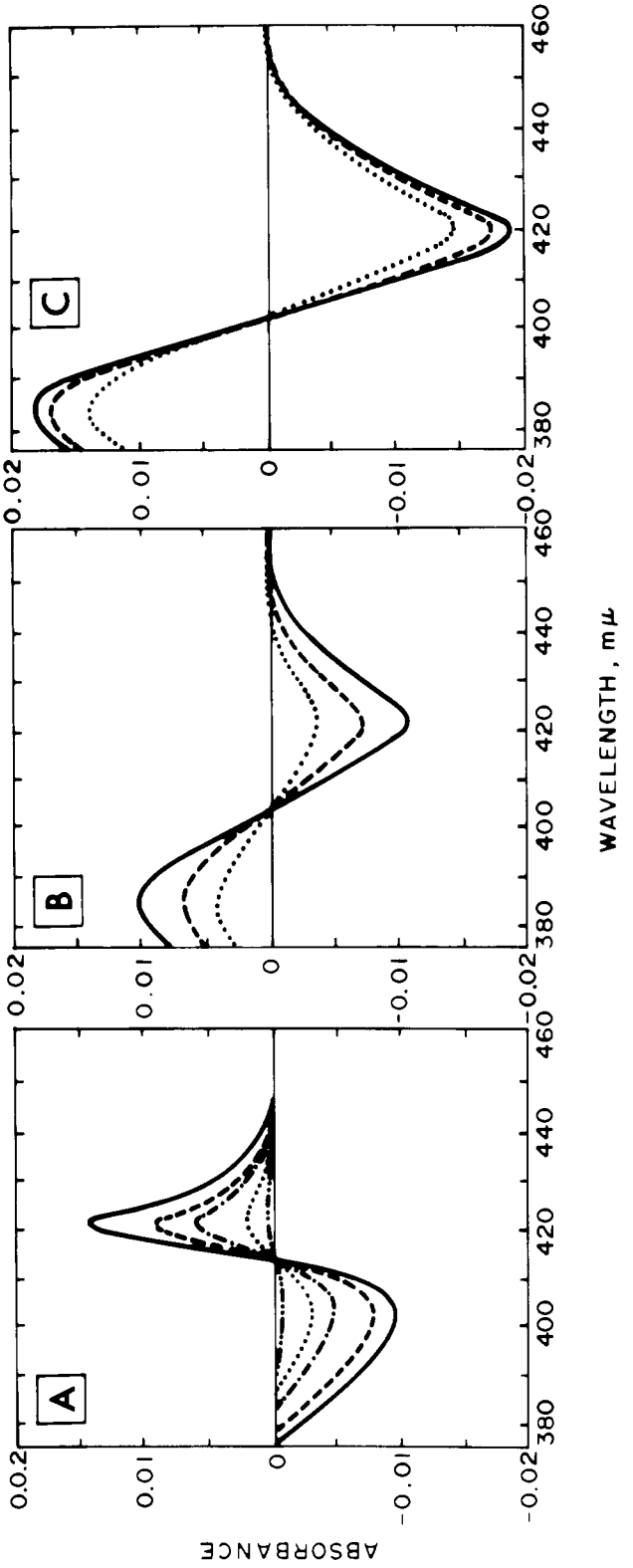

FIGURE 5: Spectral changes. (A) Produced by addition of Metopirone to the 20–40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation. For reaction conditions, see Materials and Methods. Final concentration (μmoles/l.) of Metopirone: 0.1 (— · · —), 1.0 (· · · ·), 20 (— · —), 200 (----), and 400 (—). (B and C) Effect of Metopirone on the spectral changes produced by addition of deoxycorticosterone to the 20–40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation. For reaction conditions, see Materials and Methods. (B) Deoxycorticosterone final concentration 2.4 μmoles/l.; Metopirone final concentration, μmole/l.: 0 (—), 0.1 (----), and 0.2 (· · · ·). (C) Deoxycorticosterone final concentration 21.8 μmoles/l.; Metopirone final concentration, μmole/l.: 0 (—), 0.1 (----), and 0.2 (· · · ·).

That Metopirone interferes with substrate deoxycorticosterone interaction with cytochrome P-450 is also demonstrated by the effect of the inhibitor on the substrate-induced spectral changes in cytochrome P-450. Metopirone diminishes the magnitude of the difference spectrum produced by addition of deoxycorticosterone to the cytochrome P-450 enzyme fraction. Metopirone inhibition of the substrate-induced difference spectrum is partially overcome by an increase in substrate deoxycorticosterone concentration, establishing that the inhibition is competitive with deoxycorticosterone. A similar interference by Metopirone of the difference spectrum produced on interaction of deoxycortisol with beef adrenal mitochondria has been reported in abstract form by Wilson *et al.* (1967).

As the concentration of Metopirone in the incubation mixture is increased, an additional effect on cytochrome P-450 is observed. The recovery of cytochrome P-450 is decreased slightly from that observed at low Metopirone concentrations to a level that is no longer affected by further increases in Metopirone concentration. Thus there appears to be a rapid conversion of the hemoprotein to a stable form that is not affected by incubation at 37°. Alternatively, the loss of absorbance at 450 mμ may indicate that high concentrations of Metopirone alter the conformation of cytochrome P-450 in a manner that diminishes the extinction coefficient of the carbon monoxide–hemoprotein complex. That Metopirone at high concentrations produces conformational changes in cytochrome P-450 is indicated by the ability of the inhibitor to induce spectral changes in the hemoprotein. The spectral changes induced by Metopirone are similar to those produced by organic solvents in microsomal preparations (Imai and Sato, 1967). Organic solvents such as methanol, ethanol, or 2-propanol, in concentrations from 3 to 5%, produce difference spectra exhibiting an absorption maximum at 420 mμ and a trough at 390 mμ. The solvent concentrations producing these difference spectra are of the same order of magnitude as those effecting changes in protein conformation through a disturbance of hydrophobic interactions. We submit that the spectral changes produced by high Metopirone concentrations result from a similar disturbance. This additional effect of Metopirone on cytochrome P-450 occurs at concentrations of the inhibitor higher than those required to inhibit the 11β-hydroxylation reaction. Hence it is unlikely that this effect of Metopirone on cytochrome P-450 is related to its inhibition of the 11β-hydroxylase.

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