Biochemistry

 Volume 8, Number 4 April 1969

Effects of Adrenal Mitochondrial Lipid on the Reduced Nicotinamide–Adenine Dinucleotide Phosphate Supported 11β-Hydroxylation of Deoxycorticosterone. Interaction of Phospholipid with Cytochrome P-450*

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ABSTRACT: The effects of acetone-extracted adrenal mitochondrial lipid on the reduced nicotinamide–adenine dinucleotide phosphate supported 11β-hydroxylation of deoxycorticosterone has been studied with an 11β-hydroxylase prepared from an acetone powder of beef adrenal mitochondria. The mitochondrial lipid was found to enhance the rates of both 11β-hydroxylation and reduced nicotinamide–adenine dinucleotide phosphate oxidation. Reduced nicotinamide–adenine dinucleotide phosphate oxidation was also enhanced by members of the naphthoquinone group, namely, menadione and 1,4-naphthoquinone, in a manner analogous to the mitochondrial lipid. However, benzoquinones did not stimulate the rate of reduced nicotinamide–adenine dinucleotide phosphate oxidation. While the acetone-extracted mitochondrial lipid contained the quinone coenzyme Q_{10}, no evidence was obtained showing that this compound was the lipid component responsible for enhancement of the rate of reduced nicotinamide–adenine dinucleotide phosphate oxidation. Although menadione, like mitochondrial lipid, stimulated the rate of reduced nicotinamide–adenine dinucleotide phosphate oxidation it did not enhance the rate of 11β-hydroxylation. Indeed, when reduced nicotinamide–adenine dinucleotide phosphate was rate limiting, menadione inhibited the 11β-hydroxylase reaction. It was demonstrated that menadione could accept electrons from adrenodoxin reductase, a component of the 11β-hydroxylase pathway, and thus compete with the 11β-hydroxylase for electrons from reduced nicotinamide–adenine dinucleotide phosphate. The factor present in the adrenal mitochondrial lipid that stimulates the rate of 11β-hydroxylation was precipitated by acetone at −20°, indicating that the factor was a phospholipid. The rate of 11β-hydroxylation was also stimulated by asolectin, a soybean phospholipid preparation. The stimulatory action of the mitochondrial lipid on 11β-hydroxylation was attributed to its effect on cytochrome P-450, the oxygen-activating and substrate-binding component of the 11β-hydroxylase. Mitochondrial lipid stabilized the hemoprotein, preventing decomposition of cytochrome P-450 to cytochrome P-420 during incubation at 37°. The lipid factor producing this stabilization was precipitated from acetone at −20°. Asolectin also stabilized cytochrome P-450. The stabilization of cytochrome P-450 by the adrenal mitochondrial phospholipid results in an enhanced rate of 11β-hydroxylation.

The present studies derive from the previous observation in this laboratory that the 11β-hydroxylation of deoxycorticosterone by an enzyme system extracted from an acetone powder of beef adrenal mitochondria is inhibited by dicumarol (Williamson and O'Donnell, 1967a). Numerous enzymatic reactions have been described in which dicumarol inhibits the quinone-dependent aerobic oxidation of NADH or NADPH (Williams *et al.*, 1959; Wosilait, 1960; Lester and Smith, 1961; Ernster *et al.*, 1962; Kamin *et al.*, 1965; Hall and Crane, 1967; Salach *et al.*, 1967). The absolute requirement of the steroid 11β-hydroxylase for NADPH (Sweat and Lipscomb, 1955; Grant, 1956) and the inhibition of this reaction by dicumarol may indicate the involvement of a quinone (vitamin K or coenzyme Q series) in the 11β-hydroxylase electron transport pathway (Omura *et al.*, 1965, 1966).

Cytochrome P-450, the oxygen-activating and steroid-binding component of the 11β-hydroxylase (Omura *et al.*, 1965; Wilson *et al.*, 1965; Cooper *et al.*, 1965; Cammer and Estabrook, 1967), is closely associated with phospholipid. The hemoprotein is degraded by

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phospholipase (Omura and Sato, 1964a,b) and by detergents and organic solvents which remove lipid (Mason *et al.*, 1965; Imai and Sato, 1967; Ichikawa and Yamano, 1967a). Removal of the phospholipid moiety of cytochrome P-450 converts the hemoprotein into cytochrome P-420 with a concomitant loss of enzymatic activity. Ichikawa and Yamano (1967a) suggest that hydrophobic bonding, in particular the interaction of the lipid and hemoprotein moieties of cytochrome P-450, is essential for maintaining its unique spectral and enzymatic properties.

Since essentially all the quinones (Pumphrey and Redfearn, 1960) and phospholipids (Lester and Fleischer, 1959, 1961; Lester and Smith, 1961) present in mitochondria are extracted by acetone, the 11β-hydroxylase prepared from an acetone powder of beef adrenal mitochondria would be almost devoid of these compounds. Hence the adrenal mitochondrial acetone extract, and its quinone and phospholipid components, were examined for their roles in steroid 11β-hydroxylation and NADPH oxidation, and for their effects on mitochondrial cytochrome P-450.

Materials and Methods

Chemicals. Deoxycorticosterone,[1] purchased from Sigma Chemical Co., was purified by crystallizations from acetone–hexane. [1,2-^{3}H]Deoxycorticosterone, obtained from Tracerlab and New England Nuclear Corp., was purified by paper partition chromatography in the solvent system ligroin–propylene glycol (Savard, 1953). The purification of corticosterone, purchased from Mann Research Laboratories, has been described previously (Williamson and O'Donnell, 1967a,b). Tris, NADPH, menadione, 1,4-naphthoquinone, 1,4-toluoquinone, 1,4-benzoquinone, coenzyme Q_{10}, and asolectin were obtained from commerical sources. Dicumarol was a gift from the Wisconsin Agricultural Research Foundation and Abbott Laboratories, North Chicago, Ill.

Solvents. Acetone was refluxed for 1 hr with potassium permanganate and potassium carbonate and then distilled twice. Ethanol was refluxed for 2 hr with potassium hydroxide and zinc and then distilled twice. All other solvents (AR grade) employed were purified by distillation.

Incubation Conditions. The preparation of the beef adrenal mitochondrial acetone powder and the extraction of the 11β-hydroxylase from the acetone powder with a 0.154 M KCl solution have been previously described (Williamson and O'Donnell, 1967a). The hydroxylase was extracted immediately before use in the incubations. The lyophilized powder of sonicated adrenal mitochondria was prepared by the method described by Sharma *et al.* (1962). The twice-washed mitochondrial pellet was homogenized with four times its weight of glass-distilled water at 4°. The homogenate was then sonicated for 15 min in a Bronwill 20-kc sonic oscillator. Sonication was interrupted briefly after 5 and 10 min to allow the apparatus and sonicate to cool. The sonicate was centrifuged at 105,000g for 60 min and the supernatant was then lyophilized.

A 0.1 M solution of Tris (pH 7.4) containing 2.5×10^{-3} M $MgCl_2$ was employed as the buffer medium. Reactions were carried out in 25-ml erlenmeyer flasks in a Dubnoff metabolic shaking incubator at 37°. The gas phase was air. The order of addition of reaction components was as follows: deoxycorticosterone, Tris-$MgCl_2$ buffer, enzyme preparation, a quinone, mitochondrial lipid (or asolectin), dicumarol, and finally NADPH.

Deoxycorticosterone was added to each incubation flask in methanol solution, the methanol was evaporated under nitrogen, and the steroid was redissolved in propylene glycol. The preparation of the dicumarol solution has been described previously (Williamson and O'Donnell, 1967a). The quinones were added to incubation flasks in 95% ethanol solution. The acetone-extracted adrenal mitochondrial lipid was added to incubations after the following procedure. The acetone was removed from the fraction under reduced pressure at a temperature below 40°. Water was removed by azeotropic distillation with absolute ethanol and the dried material, after being ground into a fine powder with a mortar and pestle, was suspended in a 0.1 M Tris buffer solution (pH 7.4) containing 2.5×10^{-3} M $MgCl_2$. The suspension was then sonicated in a Bronwill 20-kc sonic oscillator for 20 min to disperse the lipid material. Such treatment tends to orient the lipids to form micelles (Lester and Smith, 1961; Fleischer and Klouwen, 1961). A suspension of the commercial phospholipid preparation asolectin was prepared in a similar manner. The lipid suspensions were prepared immediately prior to addition to the reaction mixtures.

An equivalent amount of the appropriate solvent was added to incubations not containing deoxycorticosterone, dicumarol, quinones, or the lipid suspensions.

Effects of Quinones, Mitochondrial Lipid, and Dicumarol on NADPH Oxidation. For studies on NADPH oxidation, reaction flasks usually contained 1.0 ml of Tris-$MgCl_2$ buffer and 2.0 ml of enzyme preparation. The quantities of quinones, mitochondrial lipid, asolectin, and dicumarol differed slightly in the various experiments but were generally as follows: menadione, 1,4-naphthoquinone, 1,4-toluoquinone, or 1,4-benzoquinone, 0.05–1.0 μmole dissolved in 0.02–0.1 ml of ethanol; 2–10 mg of mitochondrial lipid or asolectin suspended in 0.02–0.2 ml of Tris-$MgCl_2$ buffer; and 0.5 μmole of dicumarol dissolved in 0.05 ml of the ethanol–KOH–water solution (Williamson and O'Donnell, 1967a). Each reaction mixture was incubated with shaking for 5 min at 37° and then NADPH (0.7–1.0 μmole) dissolved in Tris-$MgCl_2$ buffer (0.05–0.1 ml) was added. The rate of NADPH oxidation was measured by the diminution in absorbance at 340 mμ against a reference blank containing all reaction components except NADPH. A Beckman DU spectrophotometer was employed for these measurements.

[1] Trivial names used: deoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; corticosterone, 11β,21-dihydroxypregn-4-ene-3,20-dione; dicumarol, 3,3′-methylenebis(4-hydroxycoumarin); menadione, 2-methyl-1,4-naphthoquinone; coenzyme Q, 2,3-dimethoxy-5-methyl-6-multiprenyl-1,4-benzoquinone.

Effects of Menadione, Mitochondrial Lipid, and Dicumarol on 11β-Hydroxylation. Corticosterone formation was determined by the isolation and radioassay of this product after incubation of the 11β-hydroxylase with substrate [1,2-^{3}H]deoxycorticosterone. The concentrations of reaction components, incubation volumes, and incubation times varied slightly with each experiment, but, in general, were similar to those employed in the studies on the rate of NADPH oxidation. Reaction mixtures were preincubated for 8 min with shaking at 37° and NADPH dissolved in Tris-$MgCl_2$ buffer was added at zero time. Studies with [1,2-^{3}H]deoxycorticosterone showed that no conversion of the substrate into corticosterone occurred in the absence of NADPH (Williamson and O'Donnell, 1967b). After the desired incubation period a sample (1 ml) was removed from each reaction flask and pipetted into ice-cold ethyl acetate (2 ml) to terminate the reaction.

Isolation, Purification, and Measurement of Corticosterone. Each ethyl acetate arrested reaction mixture sample was extracted with ethyl acetate (four 2-ml portions), the organic solvent was evaporated under nitrogen, and 50 μg of nonradioactive corticosterone was added. The mixture was dissolved in a minimal quantity of methanol and corticosterone was resolved by paper partition chromatography in the solvent system toluene–propylene glycol (Burton *et al.*, 1951). The corticosterone zone was located by absorption of ultraviolet light, excised, eluted with methanol, and assayed for radioactivity with a Nuclear-Chicago Model 725 liquid scintillation counter operating at an efficiency of 40% for tritium. Steroid samples dissolved in methanol were pipetted into counting vials and the solvent was removed *in vacuo*. The steroid sample was then dissolved in 5 ml of toluene containing 2,5-diphenyloxazole (0.4%), 1,4-bis[2-(5-phenyloxazolyl)]-benzene (0.005%), and absolute ethanol (1%). All radioactivity assays were performed in duplicate. The quantity of eluted steroid was measured spectrophotometrically by absorption at 241 mμ in methanol.

Fractionation of the Acetone-Extracted Mitochondrial Lipid. Fractionation of the acetone-extracted lipid material was carried out as follows. The dried lipid (5 g) was extracted with 50 ml of petroleum ether (bp 40–60°) by continuous shaking of the lipid–solvent mixture for 2 hr with a Burrell wrist agitator. The suspension was then centrifuged, the supernatant was decanted, and the precipitate was reextracted with an additional 50 ml of petroleum ether. The combined petroleum ether extract was concentrated to a volume of approximately 20 ml under reduced pressure and the concentrate was poured, with stirring, into 200 ml of acetone previously chilled to −20°. After standing overnight at −20°, the precipitate was removed by centrifugation and the supernatant was evaporated under reduced pressure. A portion of each fraction obtained throughout the procedure was examined for its effects on the rates of NADPH oxidation and 11β-hydroxylation. Each sample was dried *in vacuo* and 10 mg of the dried material was dispersed in 1.0 ml of Tris-$MgCl_2$ buffer by sonication.

Extraction and Partial Resolution of the Total Lipids of Adrenal Mitochondria. The procedure employed for the extraction and separation of adrenal mitochondrial lipids was similar to the method described by Crane *et al.* (1959) for the isolation of coenzyme Q from beef heart mitochondria.

Mitochondria (70 g wet weight), obtained from 1000 g of beef adrenals, were suspended in a 0.25 M solution of sucrose to give a total volume of 150 ml. The mitochondrial suspension was poured into 1500 ml of an ethanol–ether mixture (3:1, v/v) and the mixture was stirred continuously for 1 hr. Solids were collected in a Buchner funnel and twice reextracted with ethanol–ether by the same procedure. The combined ethanol–ether extract was concentrated under reduced pressure to a volume of approximately 250 ml. The aqueous ethanol suspension (250 ml) was then extracted with petroleum ether (three 150-ml portions) and the combined petroleum ether extract was concentrated to approximately 15 ml under reduced pressure. The concentrate was then poured, with stirring, into 300 ml of acetone; the mixture was stored at −20° overnight and then filtered in the cold to remove precipitated phospholipid. The filtrate was evaporated under reduced pressure and the residue redissolved by warming in 20 ml of absolute ethanol. The ethanol solution was stored overnight at −20° and the white crystalline material (mostly cholesterol) obtained was removed by centrifugation.

An equivalent percentage (4%) of each fraction obtained throughout this procedure was evaporated to dryness under nitrogen and each residue was dissolved in 95% ethanol (20 ml). A portion (0.05 ml) of each ethanol solution was then examined for its effect on the rate of NADPH oxidation as previously described.

Effects of Acetone-Extracted Mitochondrial Lipid and Asolectin on Cytochromes P-450 and P-420. Cytochromes P-450 and P-420 were measured under experimental conditions similar to those employed in the studies on 11β-hydroxylation. Contamination of the enzyme preparation by hemoglobin precluded the direct assay of the incubation mixtures for cytochromes P-450 and P-420. Hence ammonium sulfate fractionation of the incubation mixtures was carried out according to the procedure outlined by Kimura and Suzuki (1967). Cytochromes P-450 and P-420 were obtained in the fraction precipitating between 20 and 40% saturation with ammonium sulfate.

A typical reaction mixture for these studies contained: propylene glycol, 0.15 ml; enzyme preparation, 3.9 ml; and 0.15–1.2 ml of a mitochondrial lipid or asolectin suspension in Tris-$MgCl_2$ buffer (10 mg of lipid/ml). The volume of each reaction mixture was adjusted to 6.3 ml with Tris-$MgCl_2$ buffer and each mixture was incubated at 37° for 8 min. In some experiments NADPH (~3 μmoles) dissolved in Tris-$MgCl_2$ buffer (0.15 ml) was added and the incubation was then continued for 90 sec. After the incubation period solid ammonium sulfate was added to each flask to give 20% saturation and the mixture was cooled to 4°. The mixture after standing for 10 min at 4° was centrifuged at 20,000*g* for 30 min. The supernatant was decanted,

brought to 40% saturation with ammonium sulfate, allowed to stand for 10 min, and then centrifuged at 20,000*g* for 30 min. The precipitate obtained was dissolved in 0.033 M Tris buffer (1.5 ml, pH 7.4) containing 8.3×10^{-4} M $MgCl_2$ and 0.1 M KCl. The cytochromes P-450 and P-420 were then determined as follows. The hemoproteins were reduced by addition of a few crystals of sodium dithionite. The mixture was then divided equally between two cuvets of 1-cm optical path and a base line from 600 to 400 mμ was recorded with a Unicam SP 800A spectrophotometer. Carbon monoxide was then bubbled gently through the solution in the sample cuvette for 45 sec and the difference spectrum was recorded at room temperature. The cytochrome P-450 content was measured by the increased absorbance at 450 mμ relative to 500 mμ. The cytochrome P-420 content was likewise measured by the increased absorbance at 420 mμ relative to 500 mμ.

Effects of Menadione on the Rate of NADPH Oxidation and on the Reduction of Cytochromes P-450 and P-420 in the 20–40% Ammonium Sulfate Fraction of the 11β-Hydroxylase. The 20–40% ammonium sulfate fraction obtained from 4 ml of enzyme preparation was dissolved in a solution of Tris-$MgCl_2$ buffer (0.6 ml) and 0.154 M KCl (1.0 ml). Propylene glycol (0.04 ml) and menadione (72 μmoles/l., final concentration) dissolved in ethanol (0.02 ml) were added and the mixture was divided equally between two cuvets. After 5-min incubation at 25°, NADPH (0.27 μmole) dissolved in Tris-$MgCl_2$ buffer (0.01 ml) was added to the sample cuvet. Tris-$MgCl_2$ buffer (0.01 ml) was added to the reference cuvet. The rate of NADPH oxidation was measured at 340 mμ employing a Unicam SP 800A spectrophotometer and compared with the rate of NADPH oxidation in a control incubation (0.02 ml of ethanol without menadione).

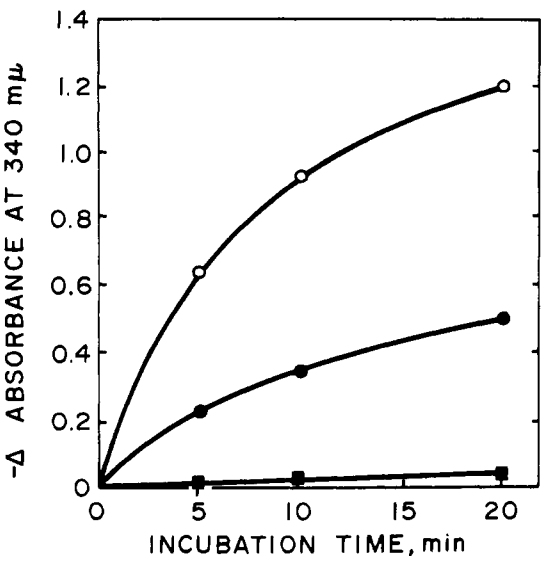


FIGURE 1: Effect of the acetone-extracted mitochondrial lipid fraction on the rate of NADPH oxidation. For reaction conditions, see Materials and Methods. A sonicated mitochondrial lipid suspension (0.02 ml, 150 mg of lipid/ml) was added to give a final lipid concentration of 0.96 mg/ml. NADPH (0.9 μmole) dissolved in Tris-$MgCl_2$ buffer (0.1 ml) was added to each reaction mixture at zero time. Contol (●); plus lipid (○); plus lipid, but with 2 ml of a 0.154 M KCl solution instead of the enzyme preparation (■).

For measurement of the extent of reduction of cytochromes P-450 and P-420 the reaction mixture described above was employed except that NADPH (0.27 μmole) was added to both the sample and reference cuvets. A base line from 500 to 400 mμ was recorded and, 3 min after addition of the NADPH, carbon monoxide was bubbled through the solution in the sample cuvet for 45 sec and the difference spectrum was recorded.

Effects of Acetone and Acetone–Water (9:1, v/v) on the Cytochrome P-450 Content of the 11β-Hydroxylase from Sonicated Adrenal Mitochondria. The lyophilized enzyme preparation (200 mg) obtained by sonication of beef adrenal mitochondria was extracted with acetone (two 10-ml portions) or with acetone–water (9:1, v/v) (two 10-ml portions) and the acetone- and acetone–water-insoluble fractions were dried *in vacuo*. The cytochrome P-450 and P-420 content of each extracted preparation and of an untreated preparation was determined as follows. A sample and reference cuvet each contained: propylene glycol (0.05 ml), Tris-$MgCl_2$ buffer (1.0 ml), and lyophilized powder (25 mg). A few crystals of dithionite were added to each cuvet. After recording a base line (500–400 mμ) carbon monoxide was bubbled through the sample cuvet for 45 sec and the difference spectrum was recorded.

Results

Effects of Adrenal Mitochondrial Lipid and Menadione on the Rate of NADPH Oxidation. Addition of a sonicated suspension of the mitochondrial acetone-extracted material to the 11β-hydroxylase results in a substantial increase in both the rate and extent of NADPH oxidation (Figure 1). Oxidation of NADPH does not occur in the presence of lipid alone; the enzyme preparation is absolutely required. Although the extent of stimulation of NADPH oxidation varied slightly in different mitochondrial lipid preparations, results similar to those shown in Figure 1 were generally observed.

A similar stimulation of the rate of NADPH oxidation is produced by the naphthoquinone, menadione (Figure 2). Menadione, at a concentration of 16 μmoles/l., produces greater than a twofold increase in the initial rate of NADPH oxidation. A series of quinones were examined and the results indicated that a significant stimulatory effect was exhibited by compounds of the naphthoquinone series, but not by the benzoquinones. 1,4-Naphthoquinone, like menadione, stimulated the rate of NADPH oxidation; 1,4-toluoquinone and 1,4-benzoquinone, members of the benzoquinone series, had only a slight stimulatory effect. Of the two benzoquinones examined, 1,4-toluoquinone was the more effective, producing a greater stimulation of the rate of NADPH oxidation at one-tenth the concentration of 1,4-benzoquinone (D. G. Williamson and V. J. O'Donnell, unpublished data).

The effect of dicumarol on the rate of NADPH oxidation stimulated by menadione or by the adrenal

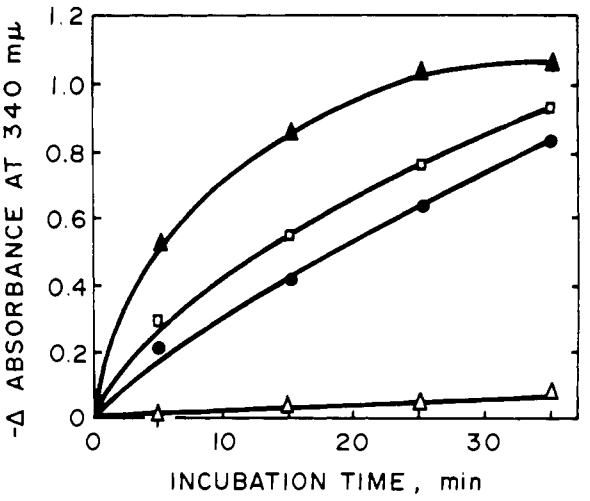


FIGURE 2: Effect of menadione on the rate of NADPH oxidation. For reaction conditions, see Materials and Methods. Menadione was added dissolved in 95% ethanol (0.1 ml). NADPH (0.7 μmole) dissolved in Tris-$MgCl_2$ buffer (0.1 ml) was added to each reaction mixture at zero time. Control (●); plus menadione, 3.13 (□) or 15.7 (▲) μmoles/l., final concentration; plus menadione but with 2 ml of a 0.154 M KCl solution instead of the enzyme preparation in a reaction mixture containing menadione, 31.3 μmoles/l. (△).

mitochondrial acetone-extracted lipid was examined (Table I). The acetone extract (3.1 mg/ml) alone produces a 4-fold increase in the initial rate of NADPH oxidation, menadione (31 μmoles/l.) produces a 2-fold stimulation, while dicumarol (155 μmoles/l.) produces a 1.4-fold stimulation. However, when dicumarol is added to the reaction mixture containing the acetone-extracted lipid there is a 2-fold increase in the rate of NADPH oxidation over that produced by addition of the lipid alone. The stimulation is even more pronounced when dicumarol is added in the presence of menadione: a 6-fold increase in the rate of NADPH oxidation over that produced by menadione alone is noted.

TABLE I: Effects of Mitochondrial Acetone-Extracted Lipid, Menadione, and Dicumarol on the Initial Rate of NADPH Oxidation.[a]

Additions	Initial Rate of NADPH Oxidation Δ Absorbance (340 mμ)/min
None	0.021
Lipid (3.1 mg/ml)	0.084
Menadione (31 μmoles/l.)	0.049
Dicumarol (155 μmoles/l.)	0.030
Lipid plus dicumarol	0.164
Menadione plus dicumarol	0.290

[a] Reaction conditions are as described in Materials and Methods. Lipid (10 mg) suspended in Tris-$MgCl_2$ buffer (0.1 ml), menadione (0.1 μmole) dissolved in ethanol (0.01 ml), and dicumarol (0.5 μmole) dissolved in the ethanol–water–KOH solution (0.05 ml) were added to appropriate reaction mixtures. NADPH (0.8 μmole) dissolved in Tris-$MgCl_2$ buffer (0.05 ml) was added at zero time.

Effects of Adrenal Mitochondrial Lipid and Menadione on the Rate of 11β-Hydroxylation. The effects of menadione and the mitochondrial acetone-extracted lipid material on 11β-hydroxylation were determined by measurement of corticosterone formation. The acetone-extracted lipid produces a 27% increase over the control in the amount of corticosterone formed after 8-min incubation (Figure 3). The inhibition of 11β-hydroxylation by dicumarol, 62% after 8-min incubation, is only slightly relieved by addition of the acetone-extracted lipid fraction. Menadione produces no increase in corticosterone formation (Figure 3); indeed, on prolonged incubation there is a decrease in the rate of 11β-hydroxylation in the presence of menadione compared to the control rate. The inhibition of the rate of 11β-hydroxylation by dicumarol is enhanced by menadione (Figure 3).

In recent studies on the electron transport system involved in steroid 11β-hydroxylation, Kimura (1966)

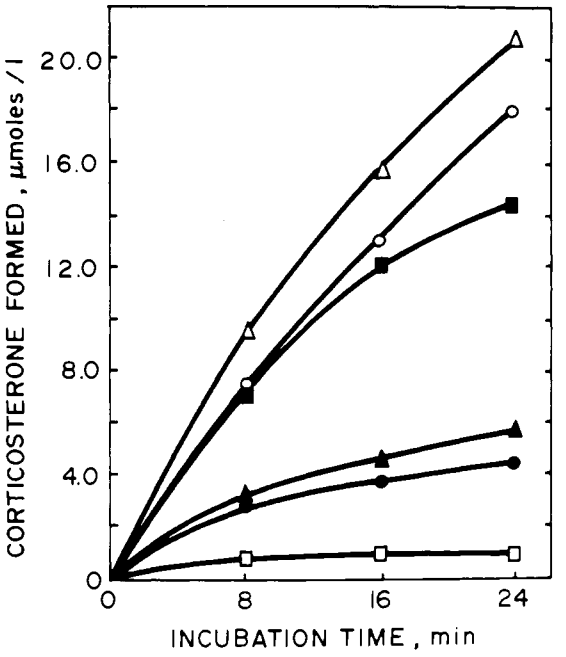


FIGURE 3: Effects of the acetone-extracted mitochondrial lipid fraction, menadione, and dicumarol on the rate of 11β-hydroxylation of deoxycorticosterone. For reaction conditions, see Materials and Methods. Appropriate reaction flasks contained: [1,2-^{3}H]deoxycorticosterone (specific activity 1.07×10^6 cpm/μmole; final concentration, 197 μmoles/l.) dissolved in propylene glycol (0.05 ml), Tris-$MgCl_2$ buffer (1.0 ml), enzyme preparation (2.0 ml), acetone-extracted lipid (10 mg) suspended in Tris-$MgCl_2$ buffer (0.1 ml), menadione (0.1 μmole) dissolved in 95% ethanol (0.02 ml), and dicumarol (0.5 μmole) dissolved in an ethanol–water–KOH solution (0.05 ml). NADPH (2.2 μmoles) dissolved in Tris-$MgCl_2$ buffer (0.08 ml) was added at zero time. Control (○); plus lipid (△); plus menadione (■); plus dicumarol (●); plus lipid and dicumarol (▲); plus menadione and dicumarol (□).

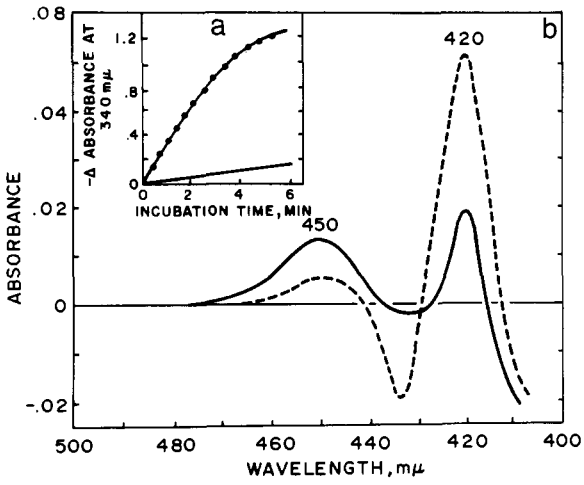


FIGURE 4: Effects of menadione on the rate of NADPH oxidation and on the extent of reduction of cytochromes P-450 and P-420 in the 20–40% ammonium sulfate fraction of the mitochondrial acetone powder enzyme preparation. For reaction conditions, see Materials and Methods. (a) Control (—); plus menadione (—●—●—●—). (b) Control (—); plus menadione (----).

has observed that menadione may function as an electron acceptor in the oxidation of NADPH by adrenodoxin reductase. Hence the menadione stimulation of the oxidation of NADPH (Figure 2) may be attributed to its role as an electron acceptor for adrenodoxin reductase. This notion was investigated.

Ammonium sulfate fractionation of the 11β-hydroxylase from an acetone powder of adrenal mitochondria yields a fraction precipitating between 20 and 40% saturation that contains the flavoprotein adrenodoxin reductase and cytochromes P-450 and P-420 (Kimura and Suzuki, 1967). However, adrenodoxin, the intermediate electron carrier between adrenodoxin reductase and cytochrome P-450 (Omura *et al.*, 1966), is not present in this fraction (Kimura and Suzuki, 1967). Hence the rate of NADPH oxidation (Figure 4a) is minimal. That slight NADPH oxidation occurs may indicate that the 20–40% ammonium sulfate fraction contains a trace amount of adrenodoxin. Addition of menadione (72 μmoles/l., final concentration) increases the rate of NADPH oxidation 21 times (Figure 4a).

The effect of menadione on the NADPH-catalyzed reduction of cytochromes P-450 and P-420 was determined by measuring the carbon monoxide difference spectra of the reduced hemoproteins in the 20–40% ammonium sulfate fraction (Figure 4b). The slow rate of oxidation of NADPH in the absence of menadione is accompanied by very little reduction of cytochromes P-450 and P-420. In the presence of menadione (Figure 4b) there is a decrease in the extent of cytochrome P-450 reduction but a substantial increase in cytochrome P-420 reduction. Thus menadione functions as an intermediate in electron transport linking adrenodoxin reductase to cytochrome P-420. The chemical reduction of cytochrome P-450 with sodium dithionite showed that this hemoprotein was present in an amount much greater than that revealed by enzymatic reduction with NADPH (D. G. Williamson and V. J. O'Donnell, unpublished data).

Examination of the Mitochondrial Acetone-Extracted Lipid Factors Affecting the Rates of NADPH Oxidation and 11β-Hydroxylation. A fractionation and partial characterization of the adrenal mitochondrial acetone-extracted lipid were undertaken in an attempt to resolve and purify the components responsible for the effects on the rates of NADPH oxidation and 11β-hydroxyla-

TABLE II: Effect of Adrenal Mitochondrial Lipid Fractions on the 11β-Hydroxylation of Deoxycorticosterone and on NADPH Oxidation.

	Corticosterone Synthesized[b]		NADPH Oxidized[c]	
Fraction Added[a]	μmole/l. per min	% of Control Act.	Δ Absorbance at 340 mμ/min	% of Control Act.
Control (no additions)	0.423	100	0.05	100
Total mitochondrial lipid	0.574	136	0.70	1400
Petroleum ether insoluble	0.456	108	0.24	480
−20° acetone insoluble	0.552	130	1.58	3160
−20° acetone soluble	0.435	103	1.26	2520
Asolectin	0.485	115	0.09	180

[a] Each fraction was suspended in Tris-$MgCl_2$ buffer at a concentration of 10 mg of lipid/ml. [b] Rate of 11β-hydroxylation of deoxycorticosterone. Each reaction flask contained: [1,2-^{3}H]deoxycorticosterone (specific activity 6.74 × 10^7 cpm/μmole) dissolved in propylene glycol (0.05 ml), final concentration 12.8 μmoles/l.; Tris-$MgCl_2$ buffer (0.55 ml); enzyme preparation (1.3 ml); and lipid suspension (0.2 ml). After incubation, NADPH (2.2 μmoles) dissolved in Tris-$MgCl_2$ buffer (0.05 ml) was added. A sample (1.0 ml) was removed from each reaction flask after a further 90-sec incubation. [c] Rate of NADPH oxidation. Each reaction flask contained: propylene glycol (0.05 ml), Tris-$MgCl_2$ buffer (0.9 ml), enzyme preparation (1.95 ml), and lipid suspension (0.2 ml). NADPH (1.0 μmole) dissolved in Tris-$MgCl_2$ buffer (0.1 ml) was added at zero time.

tion (Table II). In order to exclude the possibility that NADPH could become rate limiting and thus influence any effect of the fractions to be tested on 11β-hydroxylation, initial rates of 11β-hydroxylation and NADPH oxidation were determined. The factors stimulating both NADPH oxidation and 11β-hydroxylation are effectively extracted from the dried acetone-extracted mitochondrial lipid with petroleum ether, although some stimulatory activity remained in the petroleum ether insoluble material (Table II). The petroleum ether soluble component(s) stimulating 11β-hydroxylation are precipitable by acetone at −20°, indicating that the factor(s) producing this stimulation may be phospholipid in nature (Crane *et al.*, 1959). Moreover, stimulation of 11β-hydroxylation is achieved with asolectin, a soybean phospholipid preparation (Table II), confirming that phospholipid is responsible for this effect. The rate of NADPH oxidation is stimulated by both the −20° acetone-soluble and acetone-insoluble fractions; however, the −20° acetone-soluble fraction does not stimulate 11β-hydroxylation. These results indicate that the factor(s) stimulating 11β-hydroxylation are distinct from the factor(s) stimulating NADPH oxidation. Moreover, asolectin, which stimulates 11β-hydroxylation, produces only an 80% increase in the rate of NADPH oxidation, much less than the increases produced by the −20° acetone-insoluble fraction (3160%) and the acetone-soluble fraction (2520%).

A further examination of the lipids of beef adrenal mitochondria was undertaken in an attempt to isolate the factor(s) stimulating NADPH oxidation. The procedure employed was a modification of that described by Crane *et al.* (1959) for the isolation of coenzyme Q_{10} from beef heart mitochondria. In the present study the mitochondria obtained from 1000 g of beef adrenals were processed. The ability of each isolated fraction to stimulate the rate of NADPH oxidation was examined and the results are shown in Table III. The fractions that stimulate NADPH oxidation are also those which contain compounds of the coenzyme Q group (Crane *et al.*, 1959). The phospholipid fraction (−20° acetone insoluble) does not stimulate NADPH oxidation, indicating a more efficient separation of phospholipid from the factor(s) stimulating NADPH oxidation than was achieved previously (Table II). When the −20° acetone-soluble fraction (Table III) was evaporated to dryness *in vacuo* and redissolved in ethanol, white crystals formed on standing overnight at −20°. The crystalline material, identified as cholesterol, does not stimulate NADPH oxidation. The fractionation procedure outlined in Table III indicates that the factor(s) stimulating NADPH oxidation is extracted from mitochondria with ethanol-ether, is soluble in petroleum ether, acetone at −20°, and ethanol at −20°, and is neither phospholipid nor cholesterol.

TABLE III: Effects of Adrenal Mitochondrial Lipid Fractions on NADPH Oxidation.[a]

Lipid Fraction Added	% of Control Activity, Incubation Time (min) 5	15
Ethanol–ether soluble	457	261
Ethanol–ether insoluble	100	86
Petroleum ether soluble	484	242
Petroleum ether insoluble	111	104
−20° acetone soluble	320	247
−20° acetone insoluble	106	102
−20° ethanol soluble	340	253
−20° ethanol insoluble	103	104

[a] For reaction conditions see Materials and Methods. The lipid fraction was added in 95% ethanol (0.05 ml). NADPH (0.9 μmole) dissolved in Tris-$MgCl_2$ buffer (0.1 ml) was added at zero time.

The −20° ethanol-soluble fraction was chromatographed on a column of silica gel (Davison 923) using hexane containing increasing proportions of chloroform up to 100% for elution. Each eluate was examined for its ability to stimulate NADPH oxidation. Only the fractions eluted with chloroform stimulate the oxidation of NADPH (D. G. Williamson and V. J. O'Donnell, unpublished data). Crane *et al.* (1959) have reported that coenzyme Q_{10} is eluted from silica gel with this solvent. A portion of each column eluate was then applied to a silica gel G thin-layer chromatograph plate containing Radelin GS–115 phosphor and the chromatograph was developed with benzene. Only the silica gel column eluates that stimulated NADPH oxidation exhibited on thin-layer chromatography yellow, ultraviolet-absorbing zones having an R_F value corresponding to that of an authentic sample of coenzyme Q_{10}. These zones appeared as blue spots when sprayed with a solution of reduced methylene blue prepared according to the method of Crane and Dilley (1963). The absorption spectra of the eluates containing coenzyme Q_{10} exhibited a shoulder at 275 mμ, the region of maximum absorption of benzoquinones (Lester *et al.*, 1959). All attempts at further purification of the eluates resulted in a loss of their ability to stimulate NADPH oxidation. Moreover, addition of authentic coenzyme Q_{10} to the enzyme preparation did not stimulate NADPH oxidation.

Stimulation of the Rate of 11β-Hydroxylation by the Mitochondrial Acetone-Extracted Lipid Fraction and by Asolectin. The fractionation studies outlined in Table II indicate that phospholipid present in the acetone extract is responsible for the stimulation of 11β-hydroxylation. A comparison of the effects of the adrenal mitochondrial acetone-extracted lipid and asolectin, a plant phospholipid preparation, on the rate of 11β-hydroxylation was therefore carried out. Addition of either the acetone-extracted lipid or asolectin produced an increase in the initial rate of 11β-hydroxylation. The stimulation of the initial rate of 11β-hydroxylation as a function of lipid concentration is shown in Figure 5. Addition of increasing quantities of the acetone-extracted lipid fraction results in an increased rate of 11β-hydroxylation; a maximal stimulation of 30% is observed at a lipid concentration of 1 mg/ml. Asolectin,

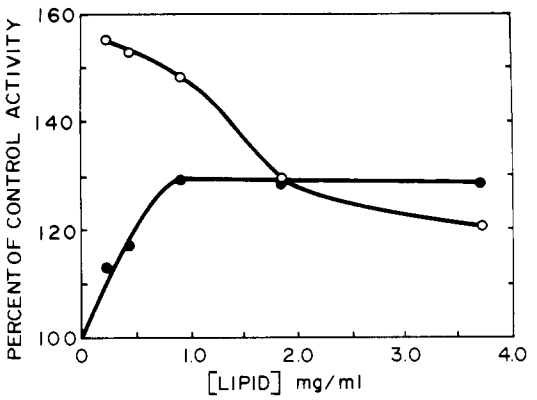


FIGURE 5: The rate of 11β-hydroxylation of deoxycorticosterone as a function of mitochondrial lipid or asolectin concentration. For reaction conditions, see Materials and Methods and Table II. [1,2-^{3}H]Deoxycorticosterone (specific activity, 4.58×10^7 cpm/μmole) dissolved in propylene glycol (0.05 ml) was added to each reaction mixture to give a final concentration of 12.8 μmoles/l. Mitochondrial lipid or asolectin (0.5, 1.0, 2.0, 4.0 and 8.0 mg) was added suspended in Tris-$MgCl_2$ buffer (0.2 ml). Mitochondrial lipid (●); asolectin (○).

however, produces 55% stimulation at the lowest concentration examined (0.25 mg/ml) but further increases in asolectin concentration cause a diminution in its stimulatory effect.

Effects of the Mitochondrial Lipid Fraction and Asolectin on Cytochromes P-450 and P-420. Studies in this laboratory have shown that substrate deoxycorticosterone stabilizes cytochrome P-450, diminishing the rate of decomposition of the hemoprotein during incubation at 37° (Williamson and O'Donnell, 1969). In the present studies it was found that the acetone-extracted mitochondrial lipid fraction increased the recovery of cytochrome P-450 in both the presence and absence of deoxycorticosterone. The acetone-extracted lipid fraction produced an increase in the recovery of cytochrome P-450 which was greater than that observed with steroid substrate. However, no additional increase in cytochrome P-450 was noted when both deoxycorticosterone and lipid were present in the reaction mixture. The factor(s) present in the acetone-extracted lipid fraction causing increased recovery of cytochrome P-450 could be precipitated by acetone at −20°.

The extent of the increase in cytochrome P-450 recovery varies as a function of the concentration of the acetone-extracted lipid fraction present in the reaction mixture (Figure 6A). A maximal increase in cytochrome P-450 is observed at a lipid concentration of 1 mg/ml. The cytochrome P-420 recovery also increases in the presence of the lipid extract (Figure 6A) although to a lesser extent than cytochrome P-450. Asolectin increases the recovery of cytochrome P-450 at lower concentrations than the lipid extract, a 2-fold increase in cytochrome P-450 being observed in the presence of asolectin at a concentration of 0.23 mg/ml (Figure 6B). However a further increase in asolectin concentration diminishes the recovery of cytochrome P-450. A similar effect of asolectin on cytochrome P-420 is observed.

In order to ascertain the mechanism whereby the acetone-extracted lipid fraction increases the recovery of cytochrome P-450 (Figure 6A), the rate of decomposition of the hemoprotein was measured in both the presence and absence of the mitochondrial lipid (Figure 7). Incubation of the enzyme preparation at 37° results in a decreased recovery of cytochrome P-450 and a corresponding increased recovery of cytochrome P-420. In the presence of the mitochondrial lipid the recovery of cytochrome P-450 is greater than in the control at all incubation times. After 4-min incubation, the cytochrome P-450 level in the reaction mixture con-

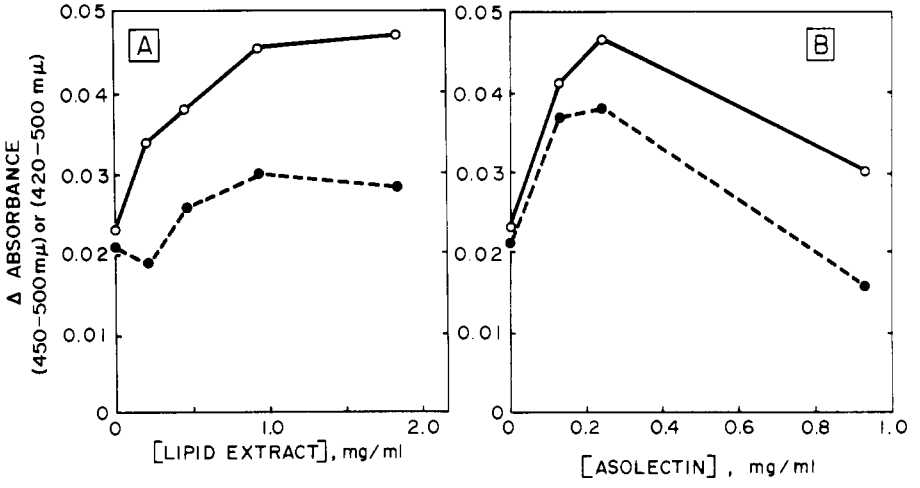


FIGURE 6: Recovery of cytochromes P-450 and P-420. (A) As a function of the acetone-extracted mitochondrial lipid concentration. For reaction conditions, see Materials and Methods; 0.15, 0.30, 0.60, or 1.2 ml of the mitochondrial lipid suspension (10 mg/ml) in Tris-$MgCl_2$ buffer was added. After 8-min incubation NADPH (3 μmoles) dissolved in Tris-$MgCl_2$ buffer (0.15 ml) was added to each reaction mixture. After a further 90-sec incubation solid ammonium sulfate to give 20% saturation was added to each reaction mixture. Cytochrome P-450 (○); cytochrome P-420 (●). (B) As a function of asolectin concentration. For reaction conditions, see Materials and Methods and part A; 0.08, 0.15, or 0.6 ml of an asolectin suspension (10 mg/ml) in Tris-$MgCl_2$ buffer was added. Cytochrome P-450 (○); cytochrome P-420 (●).

taining mitochondrial lipid decreases to the control level observed at zero time. Further incubation periods produce no additional loss of cytochrome P-450 in the presence of mitochondrial lipid.

Treatment of mitochondria with acetone–water (9:1, v/v) removes essentially all phospholipid present (Lester and Fleischer, 1959, 1961; Lester and Smith, 1961). If phospholipid functions in the maintenance of the unique spectral and enzymatic properties of cytochrome P-450, then much of the hemoprotein originally present in the intact adrenal mitochondria must be lost during preparation of the acetone powder as described in Materials and Methods. The effect of acetone on cytochrome P-450 was examined in an 11β-hydroxylase prepared by the method of Sharma *et al.* (1962). The 11β-hydroxylase is prepared by sonication of adrenal mitochondria followed by centrifugation (105,000*g*) and lyophilization. A stable enzyme preparation is thus obtained without treatment with acetone. The carbon monoxide difference spectra of cytochromes P-450 and P-420 in this preparation are illustrated in Figure 8. Cytochrome P-450 is the major hemoprotein evident, with only a trace of cytochrome P-420 being observed. The absorption maximum at 420 mμ may also be due to contamination of the preparation by hemoglobin, which also exhibits a carbon monoxide difference spectrum with an absorption maximum at 420 mμ. Treatment of the enzyme preparation with acetone does not alter this difference spectrum (Figure 8). However, if the lyophilized preparation is extracted with an acetone–water (9:1, v/v) solution, the carbon monoxide difference spectrum exhibits a decreased absorption maximum at 450 mμ and an increased absorption maximum at 418 mμ. Thus treatment with acetone–water (9:1, v/v) results in the conversion of cytochrome P-450 into cytochrome P-420, due to removal of phospholipid by this solvent combination.

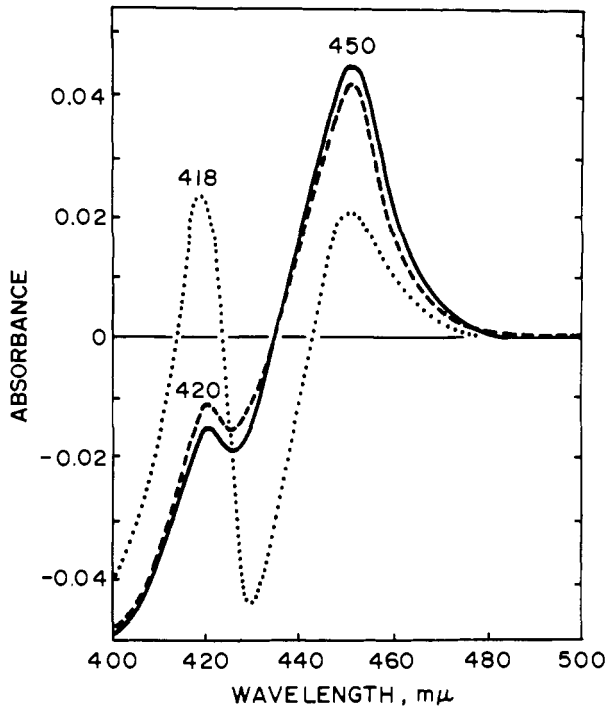


FIGURE 8: Effects of extraction with acetone or acetone–water (9:1, v/v) on the cytochrome P-450 content of the sonicated adrenal mitochondrial enzyme preparation. For reaction conditions, see Materials and Methods. Control enzyme preparation (—), acetone-extracted enzyme preparation (----), acetone-water-extracted enzyme preparation (· · · ·).

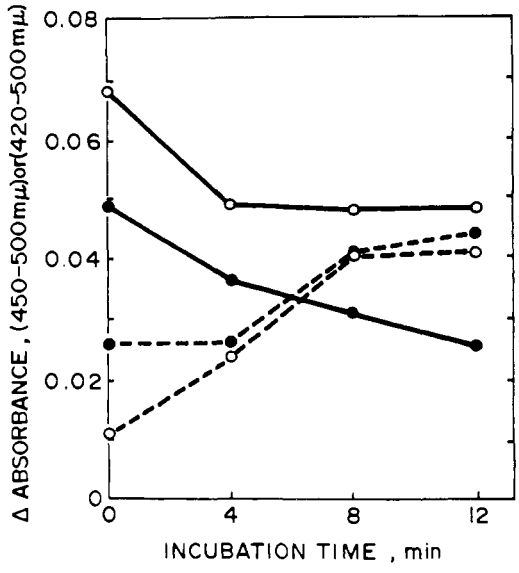


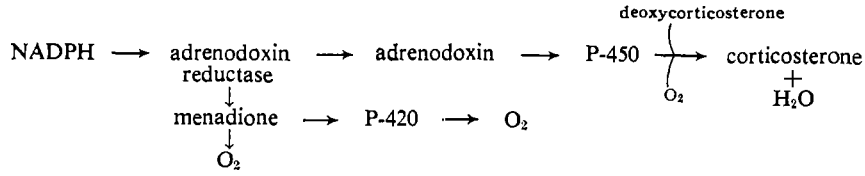
FIGURE 7: Effect of mitochondrial lipid on the rate of cytochrome P-450 disappearance. For reaction conditions, see Materials and Methods. Appropriate reaction flasks contained: propylene glycol (0.15 ml), Tris-$MgCl_2$ buffer (1.65 ml), enzyme preparation (3.90 ml), and 0.6 ml of mitochondrial lipid suspension (10 mg of lipid/ml). After incubation for periods of 0, 4, 8, or 12 min solid ammonium sulfate was added to give 20% saturation. Cytochrome P-450: control (●—●); plus mitochondrial lipid (○—○). Cytochrome P-420: control (●---●); plus mitochondrial lipid (○---○).

Discussion

The 11β-hydroxylase extracted from an acetone powder of adrenal mitochondria exhibits a naphthoquinone-stimulated oxidation of NADPH with a specificity for naphthoquinones similar to that reported for other quinone-dependent NADPH oxidases (Ernster *et al.*, 1960, 1962; Nishibayashi *et al.*, 1967). The menadione-stimulated adrenal mitochondrial NADPH oxidation is greatly enhanced by the addition of dicumarol. In contrast, the quinone reductases of the microsomal and soluble fractions of liver have been reported to be either inhibited (Ernster *et al.*, 1960, 1962) by or insensitive (Sato *et al.*, 1962; Nishibayashi *et al.*, 1963) to dicumarol.

Although menadione stimulates the rate of NADPH oxidation, it does not enhance steroid 11β-hydroxylation. Indeed, under experimental conditions where NADPH is rate limiting, menadione inhibits the 11β-hydroxylation reaction. Thus there is competition between menadione and the 11β-hydroxylase for NADPH. In the added presence of dicumarol, when the rate of NADPH oxidation is extremely rapid, this competition is even more apparent. A site for the interaction of

menadione with the 11β-hydroxylase pathway was first suggested by Kimura (1966) when he observed that adrenodoxin reductase, a constituent of the 11β-hydroxylase pathway, could utilize menadione as an electron acceptor in place of the natural acceptor, adrenodoxin. Similar observations have been made in the present studies. Moreover, the present studies suggest that menadione functions as a cofactor for the reduction of cytochrome P-420 by NADPH. However, it cannot mediate the reduction of cytochrome P-450. A summary of the proposed interaction of menadione with the 11β-hydroxylase pathway may be outlined as follows:



Electrons may be transfered from NADPH *via* adrenodoxin reductase and menadione to cytochrome P-420, which then interacts with molecular oxygen to regenerate the oxidized form of cytochrome P-420 (Omura and Sato, 1964b). Menadione is also autoxidizable (Sato *et al.*, 1962; Nishibayashi *et al.*, 1963). Thus the possibility that the oxidized form of this quinone is regenerated by interaction with molecular oxygen as well as with cytochrome P-420 cannot be disregarded. The scheme presented here illustrates the possible catalytic role of menadione in NADPH oxidation, explaining its ability to oxidize quantities of NADPH (0.6 μmole) greatly in excess of that required for the reduction of the added menadione (0.05 μmole).

The stimulation of the rate of NADPH oxidation by the acetone-extracted mitochondrial lipid fraction is similar to that observed with naphthoquinones. Both stimulatory processes are affected by dicumarol in an analogous manner. Kamin *et al.* (1965) have also reported a similar stimulation of a purified NADPH–cytochrome *c* reductase from liver microsomes by lipid extracts derived from liver and adrenocortical tissue. Whether the stimulation of NADPH oxidation by the acetone-extracted lipid fraction in the present studies is due to the presence of a quinone cannot be stated with certainty. Naphthoquinones were not detected during fractionation of the mitochondrial lipid fraction. The stimulatory activity was found to reside wholly in the silica gel column eluates containing coenzyme Q_{10}, but further attempts at purification resulted in loss of the activity. Furthermore, addition of authentic coenzyme Q_{10} to the 11β-hydroxylase under conditions employed to solubilize the quinone (*e.g.*, with Triton X-100, and as a phospholipid micelle) did not significantly enhance the rate of NADPH oxidation. Sottocasa and Crane (1965) isolated four components from the neutral lipid fraction of beef heart mitochondria that were capable of undergoing reversible oxidation–reduction. One of these components was identified as coenzyme Q_{10}; another resided in the fraction containing α-tocopherol. Of the two remaining components, one behaved chromatographically similar to known members of the vitamin K (naphthoquinone) group, while the other exhibited an absorption spectrum similar to that of coenzyme Q_{10} and was not easily separated from coenzyme Q_{10} on silicic acid column chromatography. It is possible that a similar compound is present in the lipid fraction of adrenal mitochondria and is responsible for the stimulation of NADPH oxidation. However, the fraction of the mitochondrial lipid shown to contain coenzyme Q_{10} does not stimulate 11β-hydroxylation, indicating a noninvolvement of quinones in the 11β-hydroxylase pathway.

The factor(s) stimulating 11β-hydroxylation is precipitated from acetone at −20°, a property ascribed to phospholipids (Crane *et al.*, 1959). Moreover, the rate of 11β-hydroxylation is enhanced by asolectin, a soybean phospholipid preparation. However, asolectin does not produce the extensive increase in NADPH oxidation observed with the mitochondrial lipid fraction, again indicating that the two activities of the mitochondrial lipid are attributable to two distinct factors. Imai and Sato (1959) and Sato and Imai (1961) have reported that the aromatic hydroxylation of aniline by an acetone powder of rabbit liver microsomes is enhanced by a lipid fraction extracted from the microsomes by treatment with an acetone–methanol–ether mixture.

The stimulation of the rate of 11β-hydroxylation by the acetone-extracted mitochondrial lipid may be ascribed to the effects of this fraction on cytochrome P-450, the oxygen-activating and steroid-binding component of this system (Omura *et al.*, 1965; Wilson *et al.*, 1965; Cooper *et al.*, 1965; Cammer and Estabrook, 1967). Cytochrome P-450 present in the 11β-hydroxylase of the adrenal mitochondrial acetone powder is unstable, undergoing decomposition to cytochrome P-420 during incubation at 37°. However, the acetone-extracted mitochondrial lipid increases the extent of recovery of cytochrome P-450 and diminishes its rate of decomposition. Increased recovery of cytochrome P-450 is also observed after incubation of the enzyme preparation in the presence of substrate deoxycorticosterone. Addition of both lipid and deoxycorticosterone does not stabilize cytochrome P-450 to an extent greater than that observed with the lipid alone. Thus while the protective effects of mitochondrial lipid and steroid substrate on cytochrome P-450 are similar, they are not additive. Stabilization of cytochrome P-450 by polyols such as glycerol and ethylene glycol has been reported (Ichikawa and Yamano, 1967b; Ichii *et al.*, 1967). Ichikawa and Yamano (1967b) have observed that polyols can effect a reconversion of cytochrome P-420, produced by detergent action on cytochrome P-450, into cytochrome P-450. This reconversion occurs at temperatures as low as 0°. The results of the present

studies may indicate that the mitochondrial lipid fraction produces a similar conversion of cytochrome P-420 into cytochrome P-450. In the presence of the mitochondrial lipid the recovery of cytochrome P-450 is greater than in the absence of this lipid at zero time when the enzyme preparations have been maintained at 4°. Since it has been found that cytochrome P-450 is relatively stable at low temperatures, undergoing very little decomposition (D. G. Williamson and V. J. O'Donnell, unpublished data), the increased recovery of the hemoprotein in the presence of lipid may indicate a reconstitution of cytochrome P-450 from P-420.

That the stabilizing effect of the mitochondrial lipid fraction is due to its phospholipid content is indicated by the data showing that the fraction of this lipid precipitated by acetone at −20° stabilizes the hemoprotein. Phospholipids are known to precipitate under these conditions (Crane *et al.*, 1959). Asolectin, a phospholipid preparation, also stabilizes cytochrome P-450 at low concentrations. However, as the asolectin concentration is increased, its stabilization of cytochrome P-450 is diminished. Asolectin stimulation of the rate of 11β-hydroxylation also decreases as the asolectin concentration is increased. These results indicate a relationship between the effects of asolectin on cytochrome P-450 and on 11β-hydroxylation. The diminution in these activities at the higher asolectin concentrations indicates that the phospholipid preparation may contain an inhibitory component that becomes effective at higher concentrations. Imai and Sato (1967) have observed that lysolecithin can convert cytochrome P-450 to cytochrome P-420. The asolectin preparation may contain lysolecithin, and at high concentrations of the preparation, the lysolecithin component causes destruction of cytochrome P-450 and hence diminishes the rate of 11β-hydroxylation.

The mechanism whereby phospholipids stabilize cytochrome P-450 is unclear. The hemoprotein requires phospholipid to maintain its unique spectral and enzymatic properties. Removal of phospholipid with acetone–water (9:1, v/v) converts the hemoprotein into cytochrome P-420. A similar conversion can be achieved with other organic solvents (Mason *et al.*, 1965; Imai and Sato, 1967; Ichikawa and Yamano, 1967a) and with the enzyme phospholipase (Omura and Sato, 1964a,b). Ichikawa and Yamano (1967a) suggest that the hydrophobic bonding in cytochrome P-450 plays an important role in maintaining the structure of the hemoprotein and in the binding of substrates to the cytochrome.

The significance of lipid in the biological function of the mitochondrion has been the subject of much research (Fleischer and Klouwen, 1961; Green and Fleischer, 1963; Green and Tzagoloff, 1966). Green and Tzagoloff (1966) have postulated that phospholipid micelles interact with basic proteins such as cytochrome *c* forming stable complexes. Cytochrome P-450 may well be such a complex. The addition of mitochondrial phospholipid to the 11β-hydroxylase stabilizes cytochrome P-450, effects a reconstitution of cytochrome P-450 from cytochrome P-420, and thus enhances the rate of steroid 11β-hydroxylation.

Acknowledgment

The beef adrenal glands were kindly provided by Canada Packers Limited, Vancouver, B. C.

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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-

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