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ADRENAL CORTICAL 11β-HYDROXYLASE AND SIDE-CHAIN CLEAVAGE ENZYMES. REQUIREMENT FOR THE A- OR B-PYRIDYL RING IN METYRAPONE FOR INHIBITION

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Summary—The adrenal cortical enzyme systems, 11β-hydroxylase, P-450$_{11\beta}$, and the side-chain cleavage complex, P-450 scc, differ only in their cytochrome P-450s. Structural modifications of metyrapone, an inhibitor of cytochrome P-450 enzyme systems, have been made to determine the requirement for the A- or B-pyridyl ring for inhibition of P-450$_{11\beta}$ and P-450 scc activities. Three new analogs of metyrapone (A-phenylmetyrapone, B-phenylmetyrapone and diphenylmetyrapone) were synthesized and evaluated as inhibitors using a crude, defatted bovine adrenal cortical mitochondrial preparation. Characterization of the mitochondrial preparation demonstrated: (1) enhancement of both activities by the addition of 15.0 μM adrenodoxin, (2) the addition of 1% ethanol decreased both activities less than 10%, and (3) the apparent K_m of deoxycorticosterone for P-450$_{11\beta}$ was 6.8 μM and the apparent K_m of cholesterol for P-450 scc was 21.6 μM. Inhibition of P-450$_{11\beta}$ and P-450 scc activities with these compounds demonstrated: (1) the B-pyridyl ring of metyrapone is required for inhibition of both activities whereas requirement for the A-ring is less stringent, and (2) the four metyrapone analogs were more selective inhibitors of P-450$_{11\beta}$ activity. These studies suggest that the A-phenyl metyrapone analog is a good candidate for further development of a selective adrenocortical radiopharmaceutical.

INTRODUCTION

The adrenal cortex has a unique enzymatic pattern that is directed towards the synthesis of various steroids from cholesterol [1, 2]. Of the enzymes in these pathways, both the 11β-hydroxylase and the side-chain cleavage enzyme systems are found almost exclusively in the adrenal cortex [1, 2]. The 11β-hydroxylase enzyme system, P-450$_{11\beta}$, can catalyze the 11β-, 18-, and 19-hydroxylations of various types of steroids [3–5], however, P-450$_{11\beta}$ activity is usually characterized with the substrate 11-deoxycorticosterone (DOC). The side-chain cleavage enzyme system, P-450 scc, catalyzes the side-chain cleavage of cholesterol to pregnenolone [2, 6]. Both enzyme systems are similar in that they are composed of: (1) adrenodoxin reductase, a flavoprotein, (2) adrenodoxin, an iron-sulfur protein, and (3) cytochrome P-450, a heme protein. However, the cytochrome P-450s of the two enzyme systems are immunologically distinct [4].

Metyrapone (Table 1), 2-methyl-1,2-di-(3-pyridyl)-1-propanone, is a pyridine derivative that has been widely used to determine pituitary ACTH reserve [2, 3, 7, 8]. Metyrapone inhibits the activity of various cytochrome P-450 requiring enzyme systems by reversibly binding to the specific cytochrome P-450's [3, 7, 9–13]. Of the four major cytochrome P-450 requiring enzyme systems within the adrenal cortex, the mitochondrial enzymes, P-450$_{11\beta}$ and P-450 scc, have been reported to be inhibited by metyrapone [3, 13–16] whereas the microsomal enzymes, the steroid 21-hydroxylase and the 17α-hydroxylase were reported not to be inhibited by metyrapone [13, 15–18]. This is apparently the case for the enzymes in the adrenal cortex of man, bovine and rat, but some species differences are suggested by the report of metyrapone binding to the adrenal cortical microsomes in the guinea pig [18]. Metyrapone, however, has been primarily shown, both *in vivo* and *in vitro*, to be more specific for the inhibition of P-450$_{11\beta}$ activity [1, 2, 3, 7].

We have been interested in developing a radioiodinated metyrapone analog that would concentrate in the adrenal cortex based on its selective inhibition of P-450$_{11\beta}$ activity [19]. A program has been undertaken to systematically alter the structure of the metyrapone molecule to probe those structural features necessary for the differential inhibition of P-450$_{11\beta}$ and P-450 scc. Another objective is to deter-

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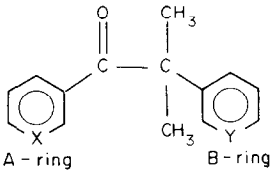
Table 1. Structure–activity relationship study: requirement for the A- or B-pyridyl ring in metyrapone for inhibition of bovine adrenal cortical mitochondrial P-450$_{11\beta}$ and B-450 scc activities (determination of the IC_{50} (μM)*)



Compound No.	Compound name	X	Y	$IC_{50}(\mu M)$* P-450$_{11\beta}$	P-450 scc
1	Metyrapone	N	N	7.8	330.1
2	A-Phenylmetyrapone	C	N	3.3	73.3
3	B-Phenylmetyrapone	N	C	64.0	392.9
4	Diphenylmetyrapone	C	C	862.9	1152.7

*IC_{50} is the μM concentration of the inhibitor to give 50% inhibition. The correlation coefficients were greater than 0.97.

mine the optimal site for incorporation of an iodine atom which still maintains this differential inhibition. In this study, we have investigated the requirement for the A- or B-pyridyl ring of metyrapone for inhibition of the two activities by systematically substituting the pyridyl rings with phenyl rings without altering the rest of the metyrapone structure (Table 1).

For this study, we wanted to use a simple preparation, containing both P-450$_{11\beta}$ and P-450 scc activities, to evaluate the metyrapone analogs. We, therefore, decided to use a crude, defatted bovine adrenal cortical mitochondrial preparation as previously described [14]. Attempts to use this preparation were unsuccessful due to little enzymatic activity in the preparation. We, therefore, report the characterization of a simple adrenocortical preparation for the rapid screening of inhibitors of P-450$_{11\beta}$ and P-450 scc activities. Using this preparation, we determined that the A-pyridyl ring of metyrapone replaced with a phenyl ring would be a good candidate for radioiodination.

Chemicals, radiochemicals and materials

The following chemicals were purchased from Sigma Chem. Co., St Louis, MO: bovine serum albumin (BSA), corticosterone, DOC, 18-hydroxy-DOC, EDTA, cholesterol, pregnenolone, progesterone, NADPH, Tween-20, Sephadex LH-20-100, Sephadex G-75-120, DEAE-cellulose and DEAE-Sephacel. *N,N*-dimethylformamide was obtained from Mallinckrodt, St Louis, MO; dithiothreitol from Calbiochem-Behring, San Diego, CA; Whatman K6F silical gel glass TLC plates, 20 × 20 cm, 250 μ, from Anspec Co., Ann Arbor, MI; Econocolumns, polypropylene, 0.8 × 4.0 cm, from Bio-Rad, Richmond, CA; and polypropylene tubes (Falcon), 12 × 75 cm, from American Scientific Products, McGaw Park, IL. Trilostane (WIN-25, 540: 2α-cyano-4α,5-epoxy-17α-hydroxy-androstane-3-one) was a generous gift from Sterling Winthrop Co., New York. Ethyl nicotinate, benzyl cyanide, tert-butanol, potassium tert-butoxide, methylene chloride, acetonitrile, propylene glycol and acetone were purchased from Aldrich Chemical Co., Milwaukee, WI. All other chemicals were the best commercially available grades.

The following radiochemicals were obtained from New England Nuclear, Boston, MA: [1,2-^{3}H(*N*)]-DOC, 41.8 Ci/ mmol; [1,2-^{3}H(*N*)]corticosterone, 40.0 Ci/mmol; [1,2-^{3}H(*N*)]cholesterol, 40.7 Ci/mmol; and [4-^{14}C]pregnenolone, 55.7 mCi/mmol. Liquifluor, the PPO-POPOP toluene concentrate, was also purchased from New England Nuclear.

Metyrapone analogs

Elemental analyses were performed by Spang Microanalytical Laboratory, Eagle Harbor, Michigan. Infrared spectra were recorded on a Beckman IR Aculab 8 spectrophotometer. Nuclear magnetic resonance spectra were recorded on a Varian EM-360A spectrophotometer. Ultraviolet spectra were recorded on a Beckman Model 35 Dual Beam UV/Visible spectrophotometer. All melting points were taken on a Laboratory Devices Mel-temp capillary melting point apparatus and are uncorrected.

Metyrapone (2-methyl-1,2-di-(3-pyridyl)-1-propanone), compound **1**, was a generous gift from Ciba-Geigy, Inc., Summit, NJ. The A-phenylmetyrapone (2-methyl-1-phenyl-2-(3-pyridyl)-1-propanone), compound **2**, was synthesized as described by Hays *et al.*[19]. Diphenylmetyrapone (2-methyl-1,2-diphenyl-1-propanone), compound **4**, was synthesized as described by Landgrebe and Kirk[20].

B-Phenylmetyrapone (2-methyl-2-phenyl-1-(3-pyridyl)-1-propanone), compound **3** was synthesized by a three step synthesis. Ethyl nicotinate, and benzyl cyanide, were treated with sodium ethoxide in absolute ethanol to yield the enol, which was hydrolysed and decarboxylated to produce the ketone as first described by Burger and Walter[21]. The hydrobromide salt of ketone (1.0 g, 0.0036 mol) was dissolved in 20 ml tert-butanol under an argon atmosphere with mechanical overhead stirring. Potassium tert-butoxide (1.23 g, 0.011 mol, 3.05 eq.) was added and the solution turned a bright yellow and was

heated to 85°C for 5 h. Methyl iodide (1.12 g, 0.49 ml, 0.0079 mol, 2.2 eq.) was added to 2 ml tert-butanol and the solution was added dropwise over a 1 h period. The reaction was heated and stirred an additional 18 h. The reaction was cooled and partitioned between water and methylene chloride. The methylene chloride layer was dried, filtered and concentrated under reduced pressure. The residue (0.71 g) was placed on a silica gel column (15 × 130 mm) and eluted with 200 ml ethyl acetate–methylene chloride (1:19 v/v). The residue (0.60 g) was taken up in 1.0 ml acetonitrile and chromatographed on a Whatman Partisil M9, 10 mm × 50 cm, ODS-2, reverse phase preparative HPLC column, eluted with water–acetonitrile (4.5:5.5, v/v) at a flow rate of 10 ml per min. 2-Phenyl-1-(3-pyridyl)-1-propanone was eluted and collected from the column first followed by the desired compound **3** with retention times of 8.32 and 11.92 min respectively. After removal of the acetonitrile, the aqueous eluant was extracted with methylene chloride. The methylene chloride layer was dried, filtered and concentrated under reduced pressure to yield the dimethyl ketone (0.176 g, 21.7%) as a clear oil. The infrared spectra (neat) was 1675 cm^{-1} (C = 0), 1384, 1362 (gem dimethyl). The ^1H nuclear magnetic resonance spectra ($CDCl_3$) was σ 1.59 (s, 6H, CH_3), 7.07 (m, 1H, aromatic) 7.32 (s, 5H, aromatic), 7.76 (m, 1H, aromatic), and 8.56 (m, 2H, aromatic). The product was converted to its bright yellow picrate salt (melting point of 127–128°C) for elemental analysis. Analysis calculated for $C_{15}H_{15}NO{\cdot}C_6H_3N_3O_7$: C, 55.51; H, 3.99; and N, 12.33. Found: C, 55.58; H, 4.05; and N, 12.38. The monomethylated ketone (1.74 g, 23.2%), as described by Napoli and Counsell [14], was also isolated.

Preparation of the crude, defatted bovine adrenal cortical mitochondrial P-450 enzymes

Adrenal cortical mitochondria were isolated, lyophilized, and defatted by a modification of the methods of Nedergaard and Canon[22] and Napoli and Counsell[14]. Bovine adrenal glands were obtained fresh from a local slaughterhouse. Cortical tissue was cleaned free of fat, capsule and connective tissue and separated from medulla. The tissue was diced and homogenized (1:10) in 0.25 M sucrose containing 10 mM potassium phosphate buffer, pH 7.40, 0.10 mM EDTA, 0.5% BSA, and 0.5 mM dithiothreitol at 4°C. The homogenate was centrifuged at 1,000 *g* for 10 min at 5°C in a Beckman J-21 C centrifuge. After filtration through cheesecloth, the supernatant was centrifuged at 10,000 *g* for 10 min. The resultant mitochondrial pellet was washed twice with homogenization buffer and the washed pellet was resuspended into 10% of the original volume of homogenization buffer without EDTA, BSA, or dithiothreitol. This suspension was lyophilized, and the evacuated flasks were flushed with argon and stored in a dessicator at −50°C. The moisture-free mitochondrial powder was homogenized in cold acetone, −20°C, (250 mg powder per 25 ml) to extract endogenous steroids and centrifuged at 20,000 *g* for 5 min at −5°C. The pellet was washed twice with the same volume of cold acetone. The precipitate was collected by vacuum filtration and dried under N_2, followed by dessication *in vacuo* over potassium hydroxide at −50°C. Immediately prior to use, the acetone powder was reconstituted by suspension into 30% propylene glycol (12.0 mg powder per 400 μl) by homogenization at 4°C. Using the method of Peterson[23] with BSA as the standard, the protein content of the acetone powder was determined to be 1.64 mg protein per 12.0 mg of the acetone powder.

Preparation of the purified adrenodoxin

Adrenodoxin was purified by a modification of the methods of Kimura and Suzuki[24] and Kimura *et al.*[25]. All procedures were carried out at 5°C unless stated otherwise. All buffer solutions were 10 mM sodium phosphate, pH 7.40, containing either 0, 170, 300 or 500 mM potassium chloride (KCl). Bovine adrenal cortical tissue, approx 0.5 kg, was homogenized and centrifuged to give a 3000 *g* supernatant in 170 mM KCl buffer. After filtering through cheesecloth and diluting with 2.4 vol of cold deionized and redistilled water, 10 g of washed DEAE-cellulose was added to the diluted supernatant, and the suspension was stirred for 18 h. The DEAE-slurry was transferred by siphon under 5 psi of N_2 to a 6.0 × 15.0 cm column where it was filtered through glass wool. After washing the packed DEAE-cellulose column with 1.0 l. of 170 mM KCl buffer, the adrenodoxin was eluted with 200 ml of 500 mM KCl buffer.

The pooled adrenodoxin was diluted with 2.0 vol of buffer with no KCl and applied onto a DEAE-Sephacel column (5.0 × 30.0 cm) which was equilibrated with 170 mM KCl buffer. After eluting the column with 1.5 column vol of the 170 mM KCl buffer, the adrenodoxin was eluted with 300 mM KCl buffer at the rate of approx 50 ml per h. Fractions of 10 ml were collected. The active fractions were pooled and concentrated to approx 10 ml by ultrafiltration in a 402-ml and then a 52-ml Diaflo cell with a YM-5 membrane (Amicon Corp., Lexington, MA) at a working pressure of 20 psi of N_2.

The concentrated enzyme solution was applied to a G-75 Sephadex column (3.0 × 100.0 cm) which was equilibrated with buffer with no KCl. The adrenodoxin was eluted with buffer at a rate of 40 ml per h. Fractions of 5 ml were collected. The active fractions were pooled, lyophilized and stored in a dessicator at −50°C. The final preparation was approx 90% homogeneous with an A_{414}-A_{276} ratio of 0.770. The yield was approx 100 mg.

Assay of P-450$_{11\beta}$ activity

This cytochrome P-450 requiring enzyme system was assayed with DOC as the substrate by a modification of the method of Sato *et al.*[3]. Using 5 ml polypropylene tubes, the reaction mixture,

0.50 ml, was modified to contain 50 mM potassium phosphate buffer, pH 7.40, 200 μM DOC (0.10 μCi [1,2-^{3}H(N)]DOC), 16 mM magnesium chloride, 1.5 mM NADPH, 4.6% propylene glycol, 15.0 μM adrenodoxin, and 300 μg of the crude, defatted mitochondrial preparation. The reaction mixture was preincubated at 37°C for 5 min, and the reaction was initiated by the addition of NADPH and incubated for various times at 37°C. Reaction controls were done in the absence of NADPH or in the presence of a heat-inactivated mitochondrial preparation where the preparation was heated at 80°C for 3–5 min. The reaction was terminated by the addition of 3.0 ml of methylene chloride containing 5 μg/ml each of DOC and corticosterone and vortexed for 30 s. After extracting and discarding the aqueous phase, the organic phase was dried down overnight at 30°C under room atmosphere.

The residue was re-extracted with 100 μl of 100% methanol and applied onto Whatman K6F silica gel glass TLC plates (20 × 20 cm plates, 250 μ, that were divided into 10 × 20 cm sections and prescored to 10 × 2.5 cm) using the AIS TLC Multi-spotter™. The plates were developed in chloroform-acetone (5:1, v/v) for 20 min at 25°C. The addition of DOC and corticosterone in the methylene chloride solution used to terminate the reaction permitted detection of the compounds under short wave ultraviolet light. Clear separation of DOC and corticosterone without overlap was obtained with the R_f values of 0.66 and 0.21 for DOC and corticosterone, respectively. The 18-hydroxy-DOC was not separated from corticosterone with this solvent system. Each zone was scraped and placed into a liquid scintillation vial. Methanol, 0.50 ml, was added to extract the ^{3}H-labeled steroid from the silica. After the addition of 10 ml of scintillant, the vials were mixed well and counted in a Beckman LS 7500 microprocessor controlled liquid scintillation system. The samples were counted for 10 min or to the preset error of 0.50%. The overall recovery for the methylene chloride extraction was 99% and for the methanol re-extraction was 72%. The amount of product formed was calculated as described where a unit of activity is defined as a pmole of DOC converted to corticosterone or 18-hydroxy-DOC per min at 37°C [3].

Assay of P-450scc activity

This cytochrome P-450 requring enzyme system was assayed by a modification of the radiometric method of Takikawa *et al.*[6] and Hanukaglu and Jefcoate[26, 27]. Using 5 ml polypropylene tubes, the reaction mixture, 0.20 ml, was modified to contain 25 mM potassium phosphate buffer, pH 7.20, 200 μM cholesterol (0.10 μCi of [1,2-^{3}H(N)]-cholesterol), 1.0 mM NADPH, 1.5% propylene glycol, 1.25% *N,N*-dimethylformamide, 0.30% Tween-20, 15.0 μM adrenodoxin, and 300 μg of the crude, defatted mitochondrial preparation. The reaction mixture was preincubated at 37°C for 5 min, and the reaction was initiated by the addition of NADPH and incubated for various times at 37°C. Reaction controls were done in the absence of NADPH or in the presence of a heat-inactivated mitochondrial preparation in which the preparation was heated at 80°C for 3–5 min. The reaction was terminated by the addition of 200 μl of 100% ethanol containing 1.0 nCi of [4-^{14}C]pregnenolone as a recovery standard. The steroids were extracted with 2.0 ml of methylene chloride. After drying, the residue was re-extracted with 200 μl of 40% methanol, and separated on Sephadex LH-20 in polypropylene minicolumns [26]. The eluted pregnenolone was collected in a liquid scintillation vial, and the methanol solution was evaporated. After the addition of 10 ml of scintillant, the vials were mixed well and counted in a Beckman LS 7500 microprocessor controlled liquid scintillation system. The samples were counted for 10 min or to a preset error of 0.50%. The ^{3}H channel was set at 0–397 and the ^{14}C channel was set at 397–655. The ^{14}C overlap into the ^{3}H channel was about 8%. The overall recovery for the methylene chloride extraction was 86% and for the methanol re-extraction was 71%. The amount of product formed was calculated as described where a unit of activity is defined as a pmole of cholesterol converted to pregnenolone per min at 37°C [6, 26, 27].

Kinetic and inhibition studies

Kinetic studies were done in the presence of 1.0% ethanol for the determination of the K_m of DOC for P-450$_{11\beta}$ activity and for the K_m of cholesterol for P-450 scc activity. The kinetic studies were analyzed by the mathematical form of the Lineweaver–Burk double-reciprocal equation. The K_m values are the mean of three separate determinations.

Stock solutions of the inhibitors were made in 100% ethanol. Inhibitors were added to the reaction mixtures of each assay prior to the addition of NADPH such that a final concentration of 1% ethanol was added. Using controls in the presence of 1% ethanol, the percent inhibition, at varying concentrations of each inhibitor, was calculated. The IC_{50} (μM) values were obtained from the linear portion of semi-logarithmic plots of percent inhibition vs concentration. Two separate determinations with five or more inhibitor concentrations were evaluated in triplicate in all IC_{50} (μM) determinations. Regression lines were calculated by least square analyses and the correlation coefficients were greater than 0.97.

RESULTS

Effect of added adrenodoxin

A crude, defatted bovine adrenal cortical mitochondrial preparation was used so that both activities might be studied without purification of the protein components of these enzyme systems. However, adrenodoxin is a loosely bound mitochondrial protein

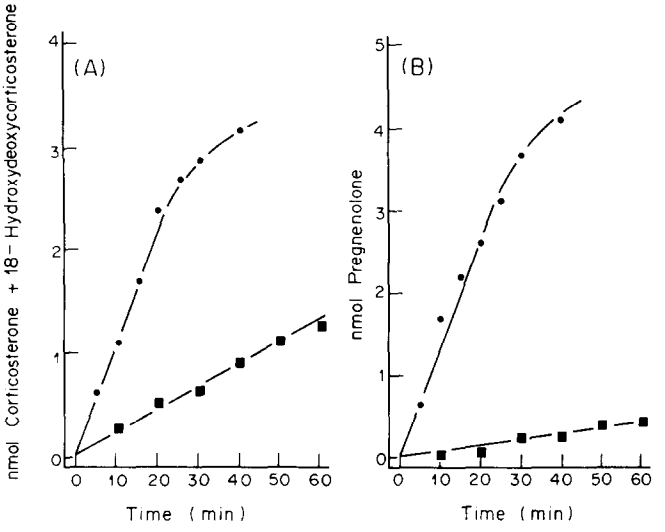


Fig. 1. Effect of added adrenodoxin to a defatted bovine adrenal cortical mitochondrial preparation on P-450$_{11\beta}$ and P-450 scc activities. A. P-450$_{11\beta}$ activity, in the absence (—■—) or presence (—●—) of 15.0 μM adrenodoxin. B. P-450 scc activity, in the absence (—■—) or presence (—●—) of 15.0 μM adrenodoxin. The points shown are the mean of triplicate determinations and are typical of three such experiments.

[24, 25] which might be lost upon preparation of the defatted mitochondria. Since this loss would lead to decreased P-450$_{11\beta}$ and P-450 scc activities and decreased sensitivity to the inhibition of these activities by the metyrapone analogs, adrenodoxin was purified to approx 90% homogeneity. The addition of 15.0 μM adrenodoxin to this preparation enhanced both P-450$_{11\beta}$ and P-450 scc activities (Fig. 1A and B). In the presence or absence of adrenodoxin, both activities were linear with time and mitochondrial protein. In this particular preparation, the specific activity of P-450$_{11\beta}$ and P-450 scc was 2,906.9 and 2,761.9 units/mg mitochondrial protein, respectively, in the presence of 15.0 μM adrenodoxin. The addition of trilostane, an inhibitor of 3β-hydroxysteroid dehydrogenase [28], to the reaction mixtures of P-450 scc did not affect the activity with time, and further metabolism of the pregnenolone formed was not detected with this preparation.

Effect of various organic solvents

Since the inhibitors to be studied are lipophilic, especially compound **4**, an organic vehicle was necessary to deliver the inhibitors into the reaction mixtures. As shown in Figs 2A and B, the addition of ethanol, *N*,*N*-dimethylformamide and *n*-propanol decreased both P-450$_{11\beta}$ and P-450 scc activities. The addition of propylene glycol was well tolerated in the P-450 scc activity assay, but it markedly decreased P-450$_{11\beta}$ activity at concentrations greater than 3%. *N*-Propanol and several other organic solvents were observed to be poorly tolerated and similarly decreased both enzymatic activities to 20% of the control levels at concentrations of 2%. Since ethanol was slightly better tolerated than *N*,*N*-dimethylformamide in both assays, it was selected as the organic vehicle. Ethanol, at 1%, gave 92 and 100% of the control assays for P-450$_{11\beta}$ and P-450 scc respectively in the absence of added organic solvent. No differences were observed in the presence or absence of 15.0 μM adrenodoxin.

Determination of the kinetic constants

As further evidence that this crude preparation could be used to study the inhibition of both activities, we determined the apparent K_m of DOC for P-450$_{11\beta}$ was 6.8 μM (Fig. 3A), and the apparent K_m of cholesterol for P-450 scc was 21.6 μM (Fig. 3B).

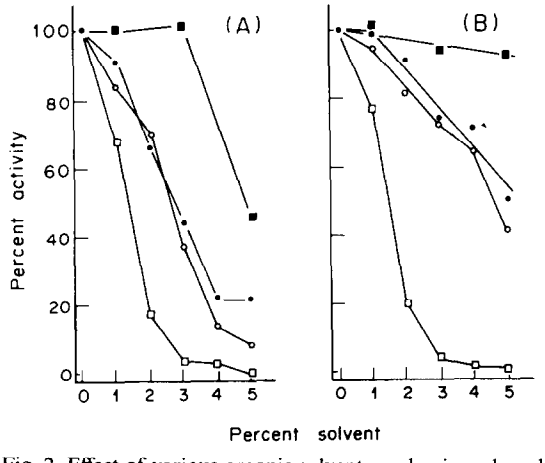


Fig. 2. Effect of various organic solvents on bovine adrenal cortical P-450$_{11\beta}$ and P-450 scc activities. Effect of propylene glycol (—■—), ethanol (—●—), dimethylformamide (—○—), and *n*-propanol (—□—) on: A. P-450$_{11\beta}$ activity, and B. P-450 scc activity. The points shown are the mean of triplicate determinations and are typical of three such experiments.

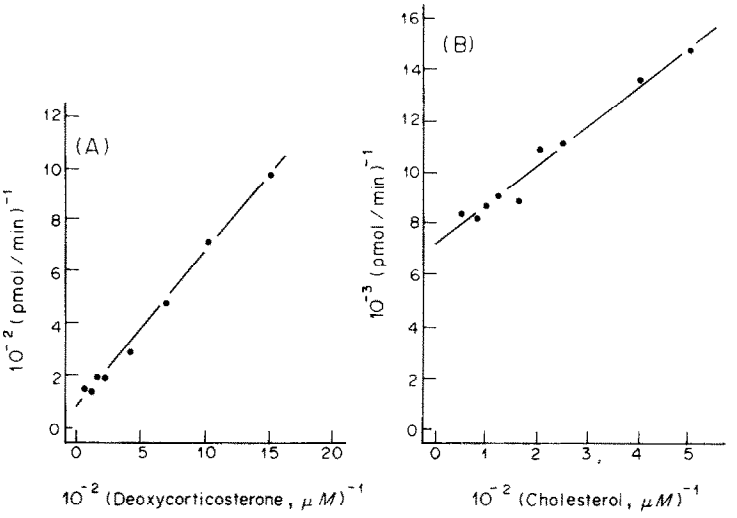


Fig. 3. Determination of the apparent K_m of DOC for P-450$_{11\beta}$ and cholesterol for P-450 scc. Standard assay conditions were used in the presence of 1% ethanol. A. Effect of varying the concentration of DOC on P-450$_{11\beta}$ activity. B. Effect of varying the concentration of cholesterol on P-450 scc activity. The points shown are the mean of duplicate determinations and are typical of three such experiments.

Although adrenodoxin has been reported to be an effector of cholesterol binding as well as an electron carrier [29], we did not observe the addition of 15.0 μM adrenodoxin to affect the K_m of cholesterol or the K_m of DOC in this system.

Inhibition studies

The inhibition of both P-450$_{11\beta}$ and P-450 scc activities by metyrapone, A-phenylmetyrapone, B-phenylmetyrapone, and diphenylmetyrapone is shown in Table 1. P-450$_{11\beta}$ activity was 1.3 to 42.3-fold more sensitive to the inhibition by the four metyrapone analogs than P-450 scc activity as shown by the IC_{50} (μM) values. Both enzyme activities require a pyridyl ring for inhibition. The B-pyridyl ring of metyrapone is required for inhibition of both cytochrome P-450s whereas the requirement for the A-pyridyl ring is less stringent. Similar results were obtained in the presence or absence of 15.0 μM adrenodoxin.

DISCUSSION

We have focused on the adrenocortical P-450$_{11\beta}$ enzyme system for the development of a radioiodinated inhibitor which would selectively concentrate into the adrenal cortex based on selective inhibition of P-450$_{11\beta}$ activity. Although metyrapone will inhibit other cytochrome P-450 requiring enzyme systems [3, 7, 9–13], radiolabeled metyrapone should concentrate in the adrenal cortex due to: (1) a higher concentration of cytochrome P-450 in the adrenal cortex than in the liver or kidney [30], and (2) apparent specificity *in vivo* and *in vitro* for P-450$_{11\beta}$ [1, 2, 3, 7]. Tissue distribution studies with tritiated metyrapone have further supported this hypothesis [31]. Since we are interested in developing a radioiodinated metyrapone analog, we initiated a program to systematically alter one region of the metyrapone molecule at a time to determine an optimal site for iodine incorporation while still maintaining a selective inhibition of P-450$_{11\beta}$ activity to that of P-450 scc activity. In this study, we synthesized three metyrapone analogs (Table 1) to determine the requirement of the A- or B-pyridyl ring for inhibition of P-450$_{11\beta}$ and P-450 scc activities.

Several studies were done to characterize the crude, defatted bovine adrenal cortical mitochondrial preparation that was used to evaluate these inhibitors. The enhancement of both activities by the addition of 15.0 μM adrenodoxin (Fig. 1A and B) suggests that considerable adrenodoxin is lost upon preparation of the defatted mitochondria. This is consistent with the observations that adrenodoxin is a loosely bound mitochondrial protein [24, 25]. The effect of organic solvents was investigated (Fig. 2A and B), not only because an organic vehicle is necessary to deliver the inhibitors into the reaction mixtures, but also because organic solvents were reported to affect hepatic cytochrome P-450 binding with hydrocarbon substrates [32]. The decreased activity observed may be the result of an interaction of the organic solvent with either or both the mitochondrial preparation and the substrates DOC and cholesterol. In one study, 12 mg of a similar mitochondrial preparation without added adrenodoxin was used to evaluate the inhibition of P-450$_{11\beta}$ activity with several metyrapone analogs where the inhibitors, as well as DOC, were delivered in ethanol to give a final concentration of 4% ethanol [14]. This concentration of ethanol drastically reduced the P-450$_{11\beta}$ and P-450 scc activities in our assays. We selected 1% ethanol, 15.0 μM adrenodoxin, and 300 μg of the crude, defatted mitochondrial preparation for these studies to minimize

the potential nonspecific binding of the inhibitors to the mitochondrial preparation and the organic interaction with the inhibitors, substrates and/or mitochondrial preparation. The observed apparent K_m of 6.8 μM for DOC for P-450$_{11\beta}$ (Fig. 3A) is similar to that reported by others [3, 14]. The observed apparent K_m of 21.6 μM for cholesterol for P-450 scc (Fig. 3B) differs from previous reports of 120 μM [6], 1.8 μM [33] and 5 μM [34] which may be due to the mitochondrial preparation used as well as the assay conditions [29].

There is limited information on the inhibition of P-450 scc by metyrapone while the evidence regarding the requirement of both pyridine rings in metyrapone for inhibition of P-450$_{11\beta}$ is not conclusive. A recent analysis of the crystal and molecular structure of metyrapone can not be used to predict which ring is important for inhibition [35], and previous studies simultaneously modified more than one structural feature of the metyrapone molecule [14, 36]. This study is a systematic evaluation of the requirement of the pyridine rings (Table 1). The inhibition of P-450$_{11\beta}$ and P-450 scc activities by these metyrapone analogs demonstrates a requirement for at least one pyridyl ring since diphenylmetyrapone is the poorest inhibitor of both activities. Since the inhibition of P-450$_{11\beta}$ activity by B-phenylmetyrapone was 8- to 19-fold less effective than A-phenylmetyrapone or metyrapone, the B-pyridyl ring of metyrapone is required for inhibition of P-450$_{11\beta}$ activity whereas the requirement for the A-pyridyl ring is not as stringent. The greater effectiveness of A-phenylmetyrapone to inhibit P-450 scc activity also suggests a requirement of the B-pyridyl ring of metyrapone for inhibition. The similar effectiveness of metyrapone and B-phenylmetyrapone to inhibit P-450 scc activity contrasts to their effectiveness to inhibit P-450$_{11\beta}$ activity. This may be due to a greater requirement for a hydrophobic region in the metyrapone molecule to bind to the active site of the cytochrome P-450 scc. The 1.3- to 42.3-fold greater sensitivity of P-450$_{11\beta}$ activity than P-450 scc activity to the inhibition of metyrapone and its A- and B-phenyl analogs may also reflect differences in the hydrophilicity of the two active-sites.

These studies suggest some of the structural requirements of metyrapone for the inhibition of P-450$_{11\beta}$ and P-450 scc activities and suggest that the A-phenylmetyrapone analog is a good candidate for radioiodination as a selective radiopharmaceutical agent for the adrenal cortex. Such an agent could be useful in evaluating patients with defective P-450$_{11\beta}$ [37, 38].

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