Steroid 11β-Hydroxylation in Beef Adrenal Cortex Mitochondria. Binding Affinity and Capacity for Specific [^{14}C]Steroids and for [^{3}H]Metyrapol, an Inhibitor of the 11β-Hydroxylation Reaction†

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ABSTRACT: The binding of deoxycorticosterone and metyrapol to cytochrome P-450 in beef adrenal cortex mitochrondria has been studied by a radioactive binding procedure. High-affinity binding sites for [^{3}H]metyrapol have only been found in the inner mitochondrial membrane in agreement with the localization of the mitochondrial P-450 in the same membrane. There was no significant binding of [^{3}H]methyrapol to the outer mitochondrial membrane, nor to the microsomes of adrenal cortex. The use of ferricyanide as a nonpenetrating electron acceptor has shown that the cytochrome P-450 involved in the 11β-hydroxylation of deoxycorticosterone is located on the matrix side of the inner mitochondrial membrane. [^{3}H]Metyrapol binding is a reversible and saturable process which proceeds rapidly towards the equilibrium state. The equilibrium changes as a function of temperature. The kinetic parameters at 0° are: $k_1 = 1.6 \times 10^5$ M^{-1} sec^{-1}, $k_{-1} = 1.2 \times 10^{-3}$ sec^{-1}, $K_d = 7.5 \times 10^{-9}$ M. The thermodynamic parameters between 0 and 31° are; $\Delta H° = -2.04$ kcal/mol, $\Delta G° = -9.6$ kcal/mol, $\Delta S° = +28$ eu. The number of [^{3}H]metyrapol sites is similar to the number of high-affinity sites found for steroids which are substrates of the 11β-hydroxylase system. This number is roughly equal to 0.5 nmol/mg of protein; it is about half the amount of mitochondrial cytochrome P-450 (1.1 nmol/mg of protein). These results support the occurrence of at least two types of cytochrome P-450 in adrenal cortex mitochondria, one specific for the 11β-hydroxylation, and another for the cholesterol side-chain cleavage. Binding of [^{3}H]metyrapol is competitively inhibited by steroids which are hydroxylated at C-11 by adrenal cortex mitochondria and conversely metyrapol inhibits the high affinity binding of [^{14}C]deoxycorticosterone. Cyclohexyl isocyanide which reacts with cytochrome P-450 at its oxygen site competitively inhibits the binding of [^{3}H]-metyrapol to the mitochondria.

The synthetic inhibitor, metyrapone[1] (2-methyl-1,2-di(3-pyridyl)-1-propanone), inhibits a number of hydroxylation reactions catalyzed by cytochrome P-450. Metyrapone has a moderately broad spectrum of action for it inhibits steroid hydroxylations at C-11β, C-18, and C-19 in adrenal cortex (Dominguez and Samuels, 1963; Sanzari and Peron, 1966; Gaunt *et al.*, 1968; Kahnt and Neher, 1971) and drug hydroxylations in liver microsomes (Leibman, 1969; Netter *et al.*, 1969; Hildebrandt, 1972; Roots and Hildebrandt, 1973a,b) and in bacteria (Peterson *et al.*, 1971; Gunsalus *et al.*, 1971), but it has no effect on the 20α- and 22-hydroxylations which are required for cholesterol side-chain cleavage in adrenal cortex mitochondria (Wilson and Harding, 1973) and on the steroid 21-hydroxylation in adrenal cortex microsomes (Dominguez and Samuels, 1963; Sweat *et al.*, 1969a). The inhibitor effect of metyrapone is accompanied by a typical modification of the difference spectrum of P-450, characterized by the appearance of a peak at 427 nm and of a trough at 410 nm and also by a shift in the low-field electron paramagnetic signal of oxidized P-450 (Wilson *et al.*, 1969).

The 11β-hydroxylation of deoxycorticosterone in adrenal cortex mitochondria is competitively inhibited by metyrapone. The spectra changes of P-450 due to the addition of deoxycorticosterone and metyrapone to adrenal cortex mitochondria and the competitive nature of the metyrapone inhibition have been interpreted as indicating that metyrapone and deoxycorticosterone bind either at a common site on the mitochondrial P-450, or at separate interacting sites (Wilson *et al.*, 1969; Williamson and O'Donnell, 1969).

The specific interaction between metyrapone and the steroid 11β-hydroxylation system in adrenal cortex mitochondria may facilitate the elucidation of the binding parameters of specific steroids and allow a better insight into the mechanism of the 11β-hydroxylation. Approximate estimates of the binding parameters for metyrapone and deoxycorticosterone have been published (Williamson and O'Donnell, 1969; Mitanie and Horie, 1969; Jefcoate and Gaylor, 1970; Whysner *et al.*, 1970) based on the spectral changes of P-450 induced by the binding of the above ligands. However, although the spectrophotometric method suggests the occurrence of interactions between metyrapone or deoxycorticosterone and P-450, it cannot be applied to an exact determination of the thermodynamic parameters. Accurate determination of these parameters requires the use of highly labeled ligands. [^{3}H]Metyrapone obtained through catalytic exchange in aqueous solution is only of limited use because of its low specific activity. Accordingly we have prepared a related compound, [^{3}H]-metyrapol, by reduction of metyrapone with [^{3}H]borohydride, and have found this to be a much more suitable analytical ligand inasmuch as it possesses the same inhibitory properties

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[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; P-450, cytochrome P-450; metyrapone, methyl-1,2-di(3-pyridyl)-1-propanone; DOC, deoxycorticosterone.

as metyrapone on the 11β-hydroxylation (Kahnt and Neher, 1971; Satre *et al.*, 1972), and has a higher specific activity.

This report describes in detail the binding properties of [^{3}H]metyrapol to adrenal cortex mitochondria and the interactions between [^{3}H]metyrapol and various steroids for binding to the 11β-hydroxylation system. The results indicate that only a portion of the mitochondrial cytochrome P-450 contained in the membrane binds metyrapol or deoxycorticosterone. Complementary studies on accessibility have shown that the part of the cytochrome P-450 specific for steroid 11β-hydroxylation is located on the matrix side of the inner mitochondrial membrane.

Experimental Section

Subcellular Preparations. Adrenal cortex mitochondria and microsomes were prepared as described previously (Satre *et al.*, 1969). Subfractionation of mitochondria into inner and outer membrane fractions was carried out by the swelling technique of Parsons and Williams (1967), adapted to adrenal cortex mitochondria (Satre *et al.*, 1969), and was monitored by measuring the activity of enzyme markers: cytochrome oxidase for the inner membrane (Appelmans *et al.*, 1955) and monoamine oxidase for the outer membrane (Wurtman and Axelrod, 1963).

Materials. [^{3}H]Metyrapol was prepared by reduction of the ketone group of metyrapone with tritiated sodium borohydride, as described in details by Satre *et al.* (1972). The specific radioactivity of the [^{3}H]metyrapol ranged between 0.9 and 1.1 Ci per mmol. Its molarity was assayed by measuring its absorbance at 261 nm in ethanol (ϵ_M = 6100). Metyrapone (metopirone, Su 4885) was a generous gift of CIBA, France. [^{14}C]Deoxycorticosterone and other labeled steroids were obtained from New England Nuclear and CEA, Saclay. The purity of [^{14}C]deoxycorticosterone and [^{3}H]metyrapol was assessed by thin-layer chromatography on silica gel G plates developed with chloroform–methanol (95:5, v/v) in the case of deoxycorticosterone and chloroform–methanol (100:16, v/v) for metyrapol; authentic nonradioactive deoxycorticosterone or metyrapol was used as controls. The purity of the compounds was greater than 98–99%.

Spectrophotometric Assays. Spectra of P-450 in mitochondrial preparations were measured at 22° in 1-cm cells in a Cary Model 15 spectrophotometer. After recording of the base line, difference spectra were obtained by addition of deoxycorticosterone or metyrapol in a minimal volume in the test cuvet and of the same volume of buffer or solvent in the reference cuvet. Total P-450 content was assayed in the presence of CO (Omura and Sato, 1967).

Binding Assays. Binding of metyrapol or metyrapone was measured in the following saline medium: 0.1 M KCl–0.01 M potassium phosphate–0.01 M Hepes (pH 7.2, final). The molar concentrations of the ligands and other specific conditions are given in the legends of the figures and tables. The particle fraction was added to initiate the reaction. Incubation was carried out in centrifuge tubes at 0° for 10 min, the binding equilibrium being reached in less than 5 min, and stopped by centrifugation at 25,000*g* for 10 min. The collection of mitochondria in a pellet was achieved in about 45 sec. The radioactivity in the supernatant and in the pellet (after digestion by 1 ml of formamide at 180°) was measured by liquid scintillation counting. Another way to stop the incubation was the filtration at 0° under vacuum through Millipore filters (pore size, 0.45 μ; diameter, 25 mm). In this case mitochondria were quickly washed with 5 ml of saline medium at 0°. The radioactivity retained on the filter was measured by liquid scintillation counting after solubilization of the filter in 1 ml of 2-methoxyethanol. Essentially similar results were obtained by both techniques. When quick sampling was required, the mitochondrial suspension was passed through a Millipore filter fitted to a syringe. An aliquot amount of the filtrate was measured by liquid scintillation counting.

Binding of [^{14}C]steroids was assayed as that of metyrapol by the centrifugation technique. Steroids were used in solution in dimethylformamide; the final concentration of dimethylformamide in the reaction medium was always less than 0.3%. Binding data were often represented by Scatchard plots (Scatchard, 1949), using the equation $B = -(B/F)K_d + N$, where B is the amount of ligand bound per milligram of protein, N is the number of binding sites per milligram of protein, K_d the molar dissociation constant, and F the molar concentration of free ligand.

Evaluation of Binding Constants for Steroid Binding. The steroid binding curve did not show a saturation level, probably due to the presence of additional nonspecific binding sites. To evaluate the number and the affinity of the specific sites, the steroid binding curves were analyzed, by assuming the presence of independent specific and nonspecific binding sites. The amount of specifically bound steroid, B_S (nmol/mg of protein), was related to the number of specific sites N_1 (nmol/mg of protein) and to the dissociation constant K_1 (molar) by the relation $B_S = N_1F/(K_1 + F)$, where F is the molar concentration of free ligand. The amount of nonspecifically bound steroid, B_{NS} (nmol/mg of protein), was given by $B_{NS} = N_2F/(K_2 + F)$, where N_2 (nmol/mg of protein) and K_2 (molar) are the amount and the dissociation constant of unspecific sites; the experimental data given below showed that F could be neglected when compared to K_2. The mass action law applied to the steroid binding equilibrium was then written: $B = B_S + B_{NS} = (N_1F)/(K_1 + F) + (N_2F/K_2)$. Using this equation a procedure based on successive approximation has been used to calculate the affinities of the two types of binding sites. To resolve the curvilinear Scatchard plot derived from the experimental binding data, an approximate value of N_2/K_2 was assumed "*a priori.*" The amount of specifically bound steroid B_S was then calculated using the relation $B_S = B - (N_2/K_2)F$ and a linear regression was performed on the set of points (B_S, B_S/F). Repeating the calculation through a set of different chosen values of N_2/K_2 permits the determination of the parameters for the high-affinity binding sites which lead to a minimal least-mean-square deviation from a straight line. This method, which led to results similar to those obtained by a graphical treatment (Rosenthal, 1967), allows a more objective evaluation of the best fit.

Results

Equilibrium Binding of [^{3}H]Metyrapol to Adrenal Cortex Mitochondria. Subcellular and Submitochondrial Distribution of the High-Affinity Metyrapol Binding Sites. A Scatchard plot of the binding of [^{3}H]metyrapol is shown in Figure 1. One may consider two regions in the curve. The first region which corresponds to low concentrations of added [^{3}H]metyrapol is characterized by a downward concavity as if low-affinity binding sites were revealed. This may be due either to the occurrence of postive interactions corresponding to an increased metyrapol affinity as metyrapol concentration increased, or to a competitive displacement of metyrapol by endogenous steroid ligands like deoxycorticosterone (see below), or possibly to a restricted diffusion of metyrapol.

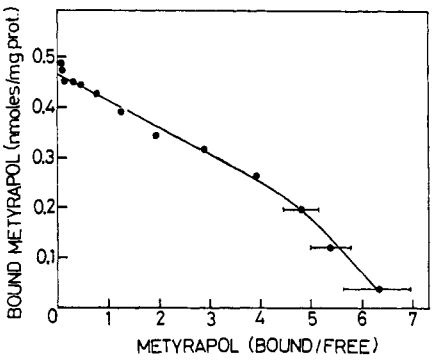

FIGURE 1: Scatchard plot representation of the binding of [^{3}H]-metyrapol to beef adrenal cortex mitochondria. Mitchondria (1.7 mg of protein) were added to a set of centrifuge tubes containing increasing concentrations of [^{3}H]metyrapol from 1.5×10^{-8} to 6.6×10^{-6} M. Total volume was 5 ml and pH 7.2. After 10 min at 0°, the incubation was stopped by centrifugation as described in the Experimental Section. The three points at the lowest [^{3}H]-metyrapol concentrations were the mean of duplicates.

The second region of the curve is linear. From its slope, a dissociation constant (K_d) for metyrapol binding can be calculated. Extrapolation to the ordinate yields the number of metyrapol sites. The number of binding sites found in nine assays ranged from 0.4 to 0.6 nmol per mg of protein and the K_d was from 10×10^{-9} to 16×10^{-9} M. The above K_d values were independent of protein concentration up to 1 mg/ml. Optimal conditions for maximal binding capacity and affinity were an isotonic saline medium buffered at pH's between 6 and 8. Outside this pH range, the affinity decreased and the number of binding sites became smaller.

In beef adrenal cortex, mitochondria appear to be the only subcellular particles endowed with a high affinity and a high capacity for metyrapol binding. The binding curve obtained with crude microsomes from beef adrenal cortex (Figure 2) requires correction for contamination with mitochondrial fragments (see Satre *et al.*, 1969), and after this is taken into account, the number of metyrapol high-affinity binding sites in beef adrenal cortex microsomes was less than 20 pmol/mg of protein. For comparison, similar binding studies were carried out with rat liver mitochondria and microsomes (Figure 3). Whereas rat liver microsomes exhibited high-affinity sites for metyrapol of the order of 50 pmol/mg of protein with a K_d of 10^{-7} M, rat liver mitochondria were virtually devoid of affinity for metyrapol. These studies clearly point to the specificity of the subcellular localization of metyrapol sites according to tissues, and also corroborate data suggesting the occurrence of different forms of P-450 having different degrees of reactivity towards metyrapol (Sweat *et al.*, 1970).

In beef adrenal cortex mitochondria, only the inner mitochondrial membrane was found to bind efficiently [^{3}H]-metyrapol, as shown in Figure 2. This is in agreement with the specific location of P-450 in the inner membrane of adrenal cortex mitochondria (Yago and Ichii, 1969; Satre *et al.*, 1969; Dodge *et al.*, 1970; Sottocasa and Sandri, 1970). The small binding capacity of outer membrane preparations from adrenal cortex mitochondria (Figure 2) was probably due to contaminant inner mitochondrial membrane fragments. Since the contamination of the outer membrane preparations with inner membrane amounted, on an average, to about 20%, on the basis of the cytochrome oxidase activity, it may be concluded that the outer mitochondrial membrane does not bind metyrapol.

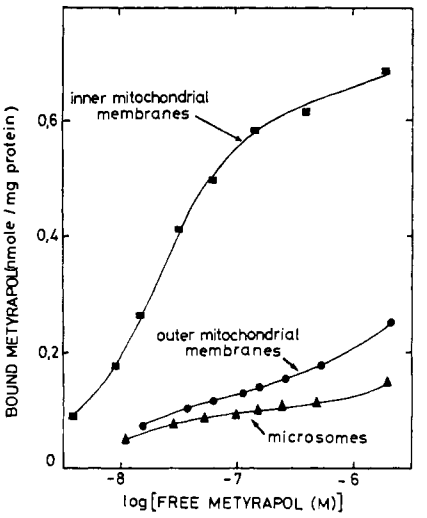

FIGURE 2: Subcellular and submitochondrial distribution of metyrapol binding sites in beef adrenal cortex. Beef adrenal cortex inner mitochondrial membranes (0.49 mg of protein), outer mitochondrial membranes (0.28 mg of protein), and microsomes (0.64 mg of protein) were incubated 10 min at 0° with increasing [^{3}H]-metyrapol concentrations (2.6×10^{-8} to 2.6×10^{-6} M). The total volume was 2 ml and pH 7.2. Incubation was stopped by centrifugation.

Thermodynamic Parameters of [^{3}H]Metyrapol Binding. The temperature dependence of [^{3}H]metyrapol binding under equilibrium conditions was tested between 0 and 31°, where no denaturation of binding sites occurs (see below). The number of binding sites was essentially independent of temperature in this range (0.45–0.55 nmol/mg of protein). From the K_d value, a free-energy change, $\Delta G°$, of −9.6 kcal/mol at 0° and of −10.5 kcal/mol at 31° was calculated (Table I). The

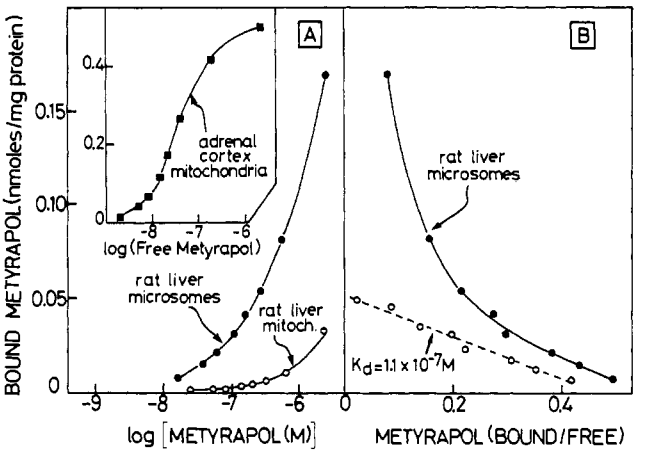

FIGURE 3: [^{3}H]Metyrapol binding to rat liver mitochondria and microsomes. Rat liver mitochondria (1.9 mg of protein), rat liver microsomes (2.1 mg of protein), and beef adrenal cortex mitochondria (2.1 mg of protein) were incubated at 0° and pH 7.2 with increasing concentrations of [^{3}H]metyrapol (from 2.4×10^{-8} to 4.8×10^{-6} M) in a total volume of 2 ml. After 10 min, the binding process was stopped by centrifugation. (A) Bound metyrapol was expressed as a function of the log of free metyrapol concentration. The binding of [^{3}H]metyrapol to beef adrenal cortex mitochondria is shown for comparison (insert). (B) Scatchard representation of the binding of [^{3}H]metyrapol to rat liver microsomes (*data shown in part A*). The curvilinear Scatchard plot was resolved into two classes of independent binding sites by the graphical method of Rosenthal (1967). Closed circles are experimental data; open circles are determined from these data and a partial plot from the low-affinity sites. The line defined by the open circles is the partial plot for the high-affinity sites.

TABLE I: Thermodynamic Parameters of Metyrapol Binding.[a]

Temp (°K)	K_d (M)	$\Delta G°$ (kcal/mol)[b]	$\Delta H°$ (kcal/mol)[c]	$\Delta S°$ (cal/mol deg)[d]
273	1.8×10^{-8}	−9.6		+27.8
			−2.3	
281	2.1×10^{-8}	−9.9		+27.9
			−1.5	
296	2.4×10^{-8}	−10.3		+28.0
			−3.4	
304	2.8×10^{-8}	−10.5		+27.9

[a] Experimental conditions are detailed in the legend of Figure 4. [b] $\Delta G° = RT \ln K_d$; $R = 1.987$ cal/(mol deg). [c] $\Delta H°$ is given between two consecutive temperatures. It was also calculated from a linear regression of $\ln K_d$ as a function of $(1/T)$ over the whole temperature range (see Figure 4). $\Delta H°$ calculated from the slope was equal to −2.04 kcal/mol. The latter value was used to calculate $\Delta S°$. [d] $\Delta S° = (\Delta H° - \Delta G°)/T$.

plot of $-\ln K_d$ *vs.* $1/T$ between 0 and 31° was shown in Figure 4. An enthalpy change, $\Delta H°$, of −2.04 kcal/mol was derived from the slope. The entropy change $\Delta S°$ was positive and equal to 28 cal mol^{-1} deg^{-1} at 0°. The negative ΔG for metyrapol binding indicates a spontaneous process resulting from the large positive entropy change. Similar positive entropy changes have been encountered in binding, by hydrophobic interactions, of testosterone and progesterone to albumin (*cf.* Westphal, 1971), and they have been explained by the displacement of structured water molecules associated with the ligand and the protein.

Kinetics of [³H]Metyrapol Binding to Adrenal Cortex Mitochondria. In kinetic binding experiments, metyrapol was used at a concentration of 1.2×10^{-7} M corresponding to the high-affinity binding portion of the Scatchard curve. Millipore filtration was preferred to centrifugation as a means to separate free from bound metyrapol since the period of incubation could be markedly reduced. As shown in Figure 5A,B, the kinetics of [³H]metyrapol binding to mitochondria was second order, and obeyed the relation $k_1 t = [1/(b - a)]x \cdot \ln [a(b - x)/b(a - x)]$, where a is the binding sites' molar concentration, x the molar concentration of bound ligand or occupied sites at time t, $(a - x)$ the free sites' molar concentration and $(b - x)$ the free metyrapol molar concentration. From the slope of the curve which is equal to $2.3/[k_1(b - a)]$, an association rate constant k_1 of 1.6×10^5 M^{-1} sec^{-1} can be calculated.

To determine the rate of dissociation of bound metyrapol, the mitochondria were first equilibrated with [³H]metyrapol, then unlabeled metyrapol was added in large excess in order to dilute extensively the unbound [³H]metyrapol and minimize the probability for rebinding of [³H]metyrapol freed by dissociation. A plot of the log of the remaining bound [³H]metyrapol *vs.* time (Figure 6) was linear for at least the first half of the process, in agreement with the first-order reaction, mitochondria–metyrapol → mitochondria + metyrapol.

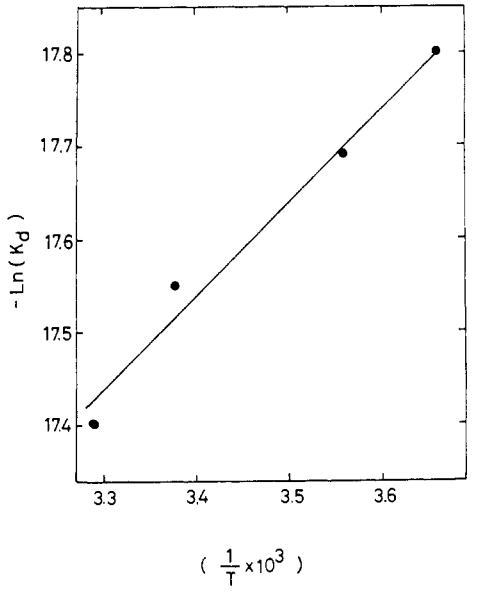

FIGURE 4: Effect of temperature on K_d value. Beef adrenal cortex mitochondria (1.7 mg of protein) were incubated for 10 min at the required temperature (0–8–23–31°) with [³H]metyrapol (1.5×10^{-8} to 6.6×10^{-6} M). The total volume was 5 ml and pH 7.2. Incubation was stopped by centrifugation. The dissociation constants K_d were obtained from Scatchard representations of binding data.

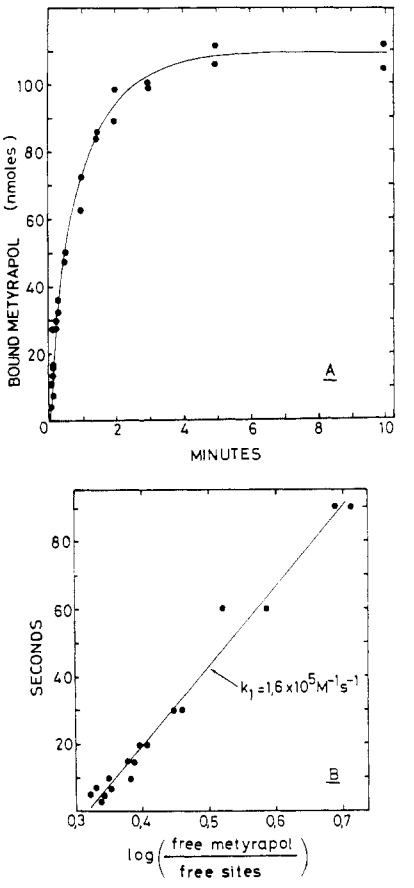

FIGURE 5: Kinetics of [³H]metyrapol binding to beef adrenal cortex mitochondria. (A) Mitochondria (0.35 mg of protein) were added at zero time to 2 ml of saline medium at 0°, pH 7.2, containing 1.2×10^{-7} M [³H]metyrapol. Incubations were stopped at a given time by quick filtration through Millipore filter fitted to a syringe. Only the free ligand was measured in the filtrate; bound [³H]metyrapol was determined by subtracting free metyrapol from the total present in the sample. (B) Analysis of the kinetic association data. From the early points shown in Figure 5A (up to 90 sec), time was plotted as a function of the log of the ratio of free metyrapol concentration to vacant sites concentration. The total concentration of binding sites was measured independently to be 5.8×10^{-8} M in a binding reaction with 2×10^{-6} M [³H]metyrapol (at this metyrapol concentration the specific sites are nearly saturated).

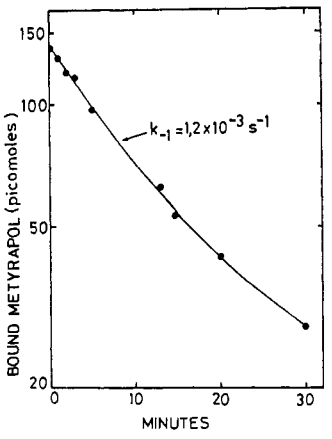

FIGURE 6: Kinetics of metyrapol dissociation from beef adrenal cortex mitochondria. Mitochondria (0.18 mg/ml) were suspended in saline medium at 0°, pH 7.2, containing 1.3 × 10^{-7} M [^{3}H]-metyrapol. After a 10-min preincubation, a zero-time control of 2 ml was sampled and filtered under vacuum through Millipore filter (see Experimental Section) immediately before adding 5 × 10^{-5} M nonradioactive metyrapol for competitive displacement. Aliquots of 2 ml were sampled at the indicated times and filtered under vacuum through Millipore filters.

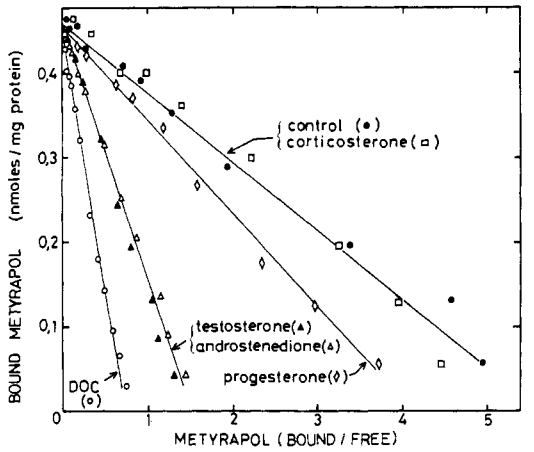

FIGURE 7: Inhibition of metyrapol binding to adrenal cortex mitochondria by steroids. Metyrapol binding was measured at 0°, pH 7.2, in saline medium. The [^{3}H]metyrapol concentrations used were from 1.5 × 10^{-8} to 4 × 10^{-6} M. Mitochondria (1.1 mg of protein) were added to initiate the reaction. The total volume was 2 ml. Incubation was carried out for 10 min and stopped by centrifugation. Steroids in dimethylformamide were added to a final concentration of 2.1 × 10^{-5} M. The control curve contained an amount of dimethylformamide equal to that introduced with the steroids (0.05% of the total volume).

The rate constant of metyrapol dissociation was calculated using the relation $k_{-1} = 0.69/t_{1/2}$, where $t_{1/2}$ is the half-dissociation time. k_{-1} was approximated to 1.2 × 10^{-3} sec^{-1}. From the kinetic constants k_{-1} and k_{+1}, a K_d value of 7.5 × 10^{-9} M was calculated in good agreement with the K_d value (10 × 10^{-9} to 16× 10^{-9} M) obtained by direct binding measurements under equilibrium conditions.

Effect of Various Steroids and Cyclohexyl Isocyanide as P-450 Ligands on the Binding of [^{3}H]Metyrapol to Adrenal Cortex Mitochondria. A number of steroids including progesterone, deoxycorticosterone, corticosterone, androstenedione, and testosterone were assayed as potential competitors of metyrapol binding on adrenal cortex mitochondria. The P-450 hydroxylation system accepts deoxycorticosterone, progesterone and androstenedione as substrates (Hayano and Dorfman, 1962). Testosterone inhibits the 11β-hydroxylation of deoxycorticosterone (Colby and Brownie, 1971), and corticosterone is the product of the 11β-hydroxylation.

As shown in Figure 7, most of these steroids inhibited competitively the binding of [^{3}H]metyrapol. The only exception was corticosterone, which at a concentration as high as 10^{-5} M did not interfere with [^{3}H]metyrapol binding. Deoxycorticosterone was the most potent inhibitor with a K_i of 3 × 10^{-6} M, followed by androstenedione and testosterone. The two latter steroids were equally competitive (K_i = 8 × 10^{-6} M) in agreement with the similarity of their inhibitory potency on the 11β-hydroxylation of deoxycorticosterone (Sharma *et al.*, 1963). Progesterone was a weaker inhibitor (K_i = 6 × 10^{-5} M).

Cyclohexyl isocyanide, which like other isocyanide derivatives reacts with P-450 at its oxygen site (Ichikawa and Yamano, 1968; Imai and Mason, 1971; Griffin and Peterson, 1971), was found to compete very efficiently with [^{3}H]metyrapol for binding to mitochondria (Figure 8). It is also shown that the affinity of adrenal cortex mitochondria for [^{3}H]metyrapol was independent of the oxidation–reduction state of the P-450.

Modification of the High-Affinity Metyrapol Binding by Alteration of Mitochondrial Membrane Components. Heat denaturation and delipidation were used to explore the chemical nature of the high-affinity binding site for metyrapol. Heat treatment was carried out as follows. Adrenal cortex mitochondria were preincubated in the standard saline medium for 3 min at various temperatures up to 80°, then incubated with a saturating concentration of [^{3}H]metyrapol (6 μM) to determine the number of binding sites remaining. As shown in Figure 9A, there was no inactivation up to 40°; then a sharp inactivation occurred between 45 and 55°, and maximal inactivation was obtained at 60°. This inactivation pattern suggests that the metyrapol binding site is protein in nature. At 60° and above there remained a small number of sites (about 0.1 nmol/mg of protein) able to bind metyrapol, but these residual thermostable sites are possibly of different chemical nature, and they were not taken into account in the kinetic study of thermal inactivation.

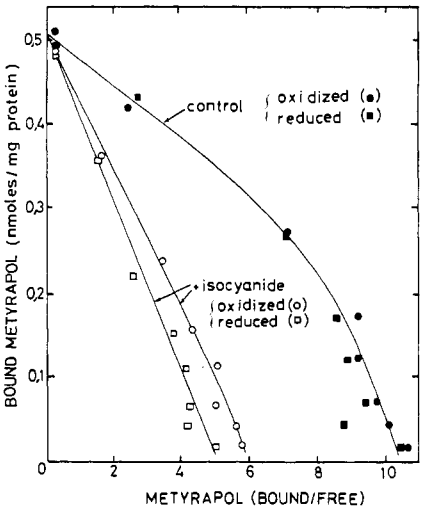

FIGURE 8: Inhibition of metyrapol binding to beef adrenal cortex mitochondria by cyclohexyl isocyanide. Adrenal cortex mitochondria (2.1 mg of protein) were added to saline medium at 0°, pH 7.2, with increasing concentrations of [^{3}H]metyrapol (2.4 × 10^{-8} to 2.4 × 10^{-6} M) in a final volume of 2 ml. After 10 min at 0°, the binding was stopped by centrifugation. This set of conditions was taken as an oxidized control (●). Other conditions included the following modifications: +5 × 10^{-3} M $Na_2S_2O_4$ (■); +10^{-5} M cyclohexyl isocyanide (○) and (5 × 10^{-3} M $Na_2S_2O_4$ + 10^{-5} M cyclohexyl isocyanide) (□).

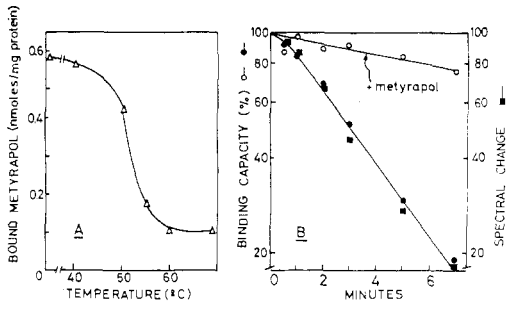

FIGURE 9: Effect of heating on the binding capacity of beef adrenal cortex mitochondria for metyrapol and on the spectral changes induced by metyrapol. (A) Samples of 1 ml of buffered saline medium (pH 7.2) were equilibrated at the indicated temperatures. Mitochondria (1.1 mg of protein) were added and after 3 min, the samples were quickly quenched with 1 ml of saline medium at 0° containing 1.2×10^{-5} M [^{3}H]metyrapol and further incubated for 10 min at 0°. Bound and free ligand were then measured after centrifugation. (B) Adrenal cortex mitochondria were added to saline medium at 51° to obtain a final concentration of 4.4 mg of protein/ml. At the indicated times, aliquots of 3 ml were quickly cooled by addition of 3 ml of saline medium at 0°. An aliquot of 1 ml was withdrawn to measure the metyrapol binding sites by adding 1 ml of 10^{-5} M [^{3}H]metyrapol (see Methods); the remaining was divided equally between two spectrophotometric cells and the spectral change (ΔOD 427–410 nm) induced by the addition of 5×10^{-6} M metyrapol to one of the cells was recorded. The bound [^{3}H]metyrapol (●) and the amplitude of the spectral change (■) induced by metyrapol were plotted as the percentage of remaining capacity after heating as compared to that of control samples processed entirely at 0°. The control values were: bound [^{3}H]-metyrapol, 0.54 nmol/mg of protein, and ΔOD 427–410 nm = 0.051. To test the protective effect of metyrapol against the denaturation by heating, the mitochondria were preincubated at 51° as above, except that 10^{-5} M unlabeled metyrapol was present in the medium. Then, the medium was cooled and [^{3}H]metyrapol was added. The incubation was stopped after 10 min by centrifugation. This incubation period allowed complete equilibration of [^{3}H]-metyrapol with the specific sites.

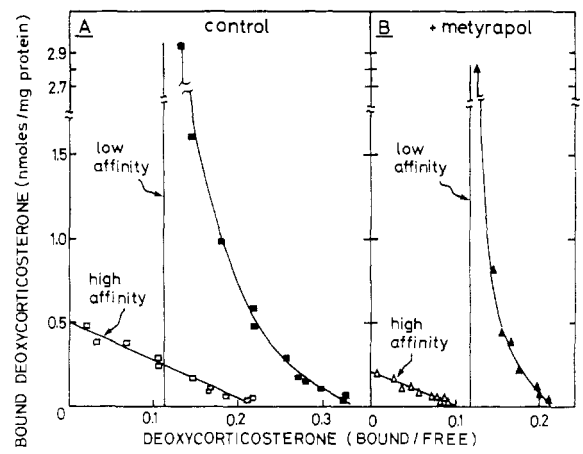

FIGURE 10: [^{14}C]Deoxycorticosterone binding to beef adrenal cortex mitochondria. Inhibition by metyrapol. Adrenal cortex mitochondria (3.2 mg of protein) were incubated at 0° with variable concentrations of [^{14}C]deoxycorticosterone (from 8×10^{-8} to 1.6×10^{-5} M). The pH was 7.2 and final volume 5 ml. After 10 min at 0°, the free and bound steroids were separated by centrifugation. The control curve is shown in part A. In part B all the tubes contained 10^{-6} M metyrapol. The Scatchard plots were resolved in two classes of binding sites as described in the Experimental Section. Filled symbols (■, ▲) are experimental data; open symbols (□, △) are calculated from the experimental data and from a partial plot of the low-affinity sites (N_2/K_2). The straight lines which join the open symbols (△, □) are the partial plots for the DOC high-affinity binding sites. The best fit was obtained with the following values: (A) control, $N_2/K_2 = 0.113$; $N_1 = 0.50$ nmol/mg of protein; $K_1 = 1.5 \times 10^{-6}$ M; linear regression coefficient = 0.981; (B) metyrapol present, $N_2/K_2 = 0.119$; $N_1 = 0.21$ nmol/mg of protein; $K_1 = 1.4 \times 10^{-6}$ M; linear regression coefficient = 0.988.

The kinetics of thermal inactivation for [^{3}H]metyrapol binding was followed at 51° (Figure 9B). A logarithmic plot of the number of remaining binding sites at a given temperature as a function of incubation times was linear, indicating the thermal inactivation process is first order and that the thermolabile sites are homogeneous with respect to their thermal sensitivity. The effect of temperature on the metyrapol-induced spectral change of cytochrome P-450 was followed in a parallel experiment and evaluated by the decrease of the spectral change. The kinetics of thermal inactivation were again first order and were virtually identical with those measured with [^{3}H]metyrapol. The similarity of thermal inactivation data obtained by the isotopic and the spectrophotometric techniques agrees with the concept that the metyrapol binding receptor in adrenal cortex mitochondria is P-450. As might be expected, metyrapol added during the heating step affords a marked protection against denaturation (Figure 9B).

The binding affinity and capacity of adrenal cortex mitochondria were practically constant from pH 6 to 8. Below pH 6, the binding capacity increased and the affinity decreased. For example, at pH 5.6 as compared to pH 7.2, the affinity decreased by 2.5-fold and the quantity bound increased by 15%. This effect of pH could be related either to the ionization state of the membrane receptor or to that of the metyrapol itself.

Delipidation of adrenal cortex mitochondria was carried out by treatment with 90% aqueous acetone at 0° (Fleischer and Fleischer, 1967). This treatment decreased the lipid phosphorus in the mitochondria from 500 to 200 natoms per mg of protein. The metyrapol thermolabile and thermostable binding sites were not modified by this delipidation, and neither was the competitive effect of DOC on metyrapol binding. These data point again to the protein nature of the metyrapol sites. Delipidation was also intended to check whether the paradoxical low-affinity binding zone in the titration curve at low concentration of metyrapol was related to endogenous steroids competing with added metyrapol. Two facts are against this hypothesis: (1) delipidation, which is expected to lower the level of endogenous steroids, does not decrease this low affinity binding; (2) if the low affinity portion of the metyrapol binding curve were due to endogenous DOC, preincubation with DOC would amplify this portion of the curve. In fact, preincubation of lipid-depleted mitochondria with DOC does not have this effect.

Inhibition of [^{14}C]Deoxcyorticosterone Binding by Metyrapol. Metyrapone decreases significantly the spectral interaction of deoxycorticosterone with mitochondrial P-450 and causes a slight shift of the spectrum toward longer wavelengths. Metyrapol behaves in the same manner (Satre, 1973). The diminished spectral interaction of deoxycorticosterone brought about by metyrapol suggested, but did not prove, that metyrapol and deoxycorticosterone compete for binding to the same site. To investigate further the nature of the competition, the effect of metyrapol on the binding of [^{14}C]deoxycorticosterone to adrenal cortex mitochondria has been examined. The data for [^{14}C]deoxycorticosterone binding can be well fitted by assuming two types of binding sites with different K_d values. This fitting results in a number of high-affinity sites of about 0.4–0.5 nmol/mg of protein with a K_d value of about 1×10^{-6} M (Figure 10A). This value is close to the K_i value found in assays where metyrapol binding was

inhibited by deoxycorticosterone (see above). On the other hand, the low-affinity sites for deoxycorticosterone are characterized by a K_d value higher than 5×10^{-3} M, and can be explained by partition of deoxycorticosterone between the medium and the membrane phospholipids.

Data in Figure 10B show that metyrapol interacts only with the deoxycorticosterone high-affinity sites. Curiously enough, whereas the inhibition of [^{3}H]metyrapol binding by deoxycorticosterone was clearly competitive (see above), the inhibition of [^{14}C]deoxycorticosterone binding by metyrapol was apparently noncompetitive: the number high-affinity sites for deoxycorticosterone was decreased without significant change in the K_d value. Such a paradoxical behavior where truly competitive inhibitors behave as noncompetitive inhibitors occurs when the inhibitor exhibits a much higher affinity than the substrate for the binding sites and the concentrations of inhibitor and binding sites are about the same (Webb, 1963; Henderson, 1972). This is the case for the binding of metyrapol to adrenal cortex mitochondria; its K_d (10^{-8} M) is 100 times less than that of deoxycorticosterone (10^{-6} M). Assays carried out with purified membrane preparations obtained from adrenal cortex mitochondria showed that metyrapol binds with high affinity only to the inner mitochondrial membrane. Only low affinity sites, probably of lipid nature, were found in the outer mitochondrial membrane. The microsomal fraction from adrenal cortex also possesses low-affinity sites.

Binding Capacity and Affinity of Adrenal Cortex Mitochondria for Steroids Undergoing Steroid 11β-Hydroxylation. Like deoxycorticosterone, the following steroids deoxycortisol, progesterone, and androstenedione are substrates of the mitochondrial 11β-hydroxylation. The binding parameters of the above steroids to the mitochondria system are listed in Table II. The high-affinity sites for these steroids all have approximately the same dissociation constants. The number of binding sites was about 0.4 nmol/mg of protein for deoxycorticosterone, deoxycortisol, or androstenedione, and twice as much for progesterone. However, the accuracy of the fitting for progesterone is questionable in view of the very lipophilic character of this steroid. That the above steroids bind with high affinity to a same site was ascertained by competition experiments. As an example; data in Figure 11 show the strict competition for specific binding between [^{14}C]DOC and androstenedione. The low-affinity binding

TABLE II: Binding Characteristics of Various Steroids to Beef Adrenal Cortex Mitochondria.[a]

	Specific Binding		Nonspecific Binding
	N_1 (nmol/mg of Protein)	K_1 (μM)	N_2/K_2
Deoxycorticosterone	0.41	0.8	0.21
Deoxycortisol	0.38	2.8	0.10
Androstenedione	0.40	2.0	0.10
Progesterone	0.83	2.6	0.75
Corticosterone	0.09	1.0	0.10

[a] Adrenal cortex mitochondria (1.6 mg of protein) were incubated at 0°, pH 7.2, with increasing concentrations of [^{14}C]steroids from 1.4×10^{-8} to 1.1×10^{-5} M. The final volume was 1.5 ml. The incubation was ended by centrifugation and the respective binding data were resolved in two classes of independent sites as described in the Experimental Section.

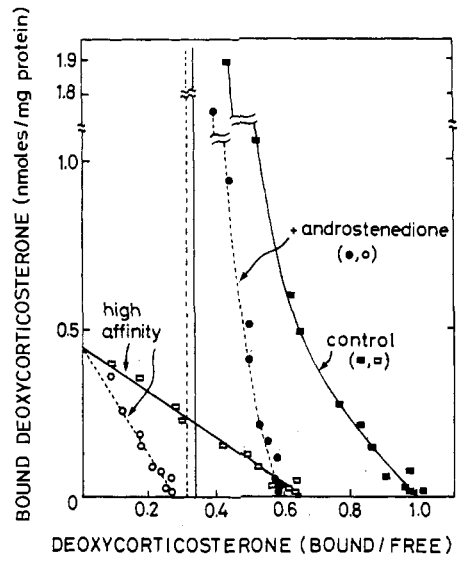

FIGURE 11: Inhibition by androstenedione of [^{14}C]DOC binding to beef adrenal cortex mitochondria. Adrenal cortex mitochondria (3.2 mg of protein) were incubated for 10 min at 0° with increasing [^{14}C]deoxycorticosterone concentrations (3×10^{-8} to 10^{-5} M) in a final volume of 2 ml, final pH 7.2. Incubation was ended by centrifugation. The Scatchard plots obtained from the binding data were resolved in two classes of sites as described in the Experimental Section. Filled symbols (■, ●) are experimental points. The lines defined by the open symbols (□, ○) are the partial plots for the DOC high-affinity binding sites. The best fit was obtained with the following values: (A) control, $N_2/K_2 = 0.345$; $N_1 = 0.45$ nmol/mg of protein; $K_1 = 1.1 \times 10^{-6}$ M (linear regression coefficient = 0.991); (B) 5.6×10^{-6} M androstenedione present, $N_2/K_2 = 0.315$; $N_1 = 0.45$ nmol/mg of protein; $K_1 = 2.5 \times 10^{-6}$ M (linear regression coefficient = 0.984).

was characterized by ratios of bound to free ligands which increased with the hydrophobicity of the steroid, as assessed by its partition coefficient between isobutyl alcohol and water (*cf.* Westphal, 1971).

The low binding capacity of adrenal cortex mitochondria for corticosterone (<0.1 nmol/mg of protein) as compared to the high binding capacity for deoxycorticosterone is in line with the movements of these steroids in the cell since deoxycorticosterone present in cytosol must be transported into the mitochondria before undergoing hydroxylation to corticosterone, and then the corticosterone must be related back into the cytosol.

Localization of the 11β-Hydroxylation System in the Inner Mitochondrial Membrane. The 11β-hydroxylation system is located in the inner mitochondrial membrane. It was shown that among various artificial electrons acceptors, ferricyanide is the most effective with the isolated mitochondrial NADPH–adrenodoxin reductase (Kimura, 1966; Kimura and Nakamura, 1971). Ferricyanide is a nonpenetrating electron acceptor which has been used for the study of the topography of the inner mitochondrial membrane in rat liver mitochondria (Klingenberg, 1970). Experiment of a similar type described in Figure 12 shows that the 11β-hydroxylation system is located on the matrix face of the inner membrane of adrenal cortex mitochondria. In this experiment, we followed the respiration of adrenal cortex mitochondria in the presence of malate. The malate-dependent respiration was inhibited by ferricyanide, probably because the ferricyanide traps the reducing equivalents from the respiratory chain at the level of cytochrome *c* (Estabrook, 1961). The stimulation of O_2 uptake by deoxycorticosterone was due to the 11β-hydroxylation of deoxycorticosterone, as corroborated by the lack of

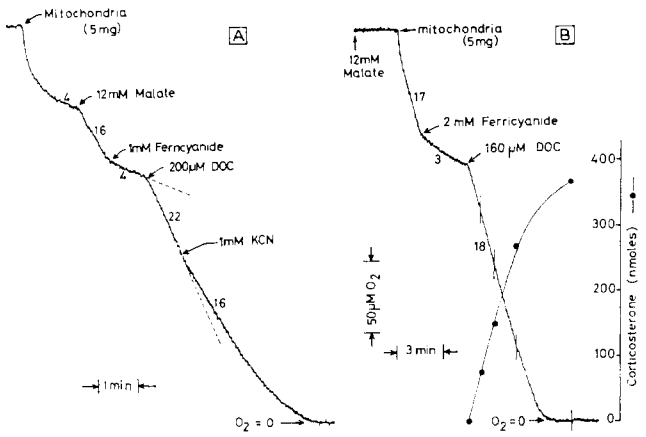

FIGURE 12: Effect of ferricyanide on the rate of oxygen uptake by adrenal cortex mitochondria. (A) Mitochondria (5 mg of protein) were added to 2 ml of the standard saline medium used for the binding experiments (see Experimental Section). The temperature was 25°. L-Malate, potassium ferricyanide, deoxycorticosterone (DOC), and potassium cyanide were added as indicated. The O_2 uptake was recorded with a Gilson oxygraph. The figures along the trace are respiration rates in nanomoles of O_2/min per mg. (B) Same experimental conditions as in (A) except that [^{14}C]deoxycorticosterone was used. 0.1-ml aliquots were sampled in the course of the incubation (vertical bars) for measurement of [^{14}C]corticosterone after thin-layer chromatography (Satre *et al.*, 1969).

effect of cyanide (Figure 12A) and the concomitant increase in corticosterone (Figure 12B). Ferricyanide did not affect the O_2 uptake induced by deoxycorticosterone, presumably because it did not gain access to the 11β-hydroxylation system in the inner membrane.

Discussion

It has been shown in a previous paper (Satre *et al.*, 1972) that metyrapol, the reduced derivative of metyrapone, behaves with the same efficiency as metyrapone for binding to adrenal cortex mitochondria and for inhibiting the 11β-hydroxylation of deoxycorticosterone into corticosterone. Because [^{3}H]metyrapol with a high specific radioactivity could be easily prepared, interaction of metyrapol with adrenal cortex mitochondria would be studied by a direct binding technique. We have found that the binding of [^{3}H]metyrapol to beef adrenal cortex mitochondria is a saturable, reversible and specific process. The kinetic data for binding and dissociation obey simple rate equations. The dissociation constant, K_d, 8×10^{-9} M, calculated from the k_{-1}/k_{+1} ratio is very close to that determined from equilibrium measurements ($10–16 \times 10^{-9}$ M). The low binding affinity of adrenal cortex microsomes for metyrapol ($K_d = 1 \times 10^{-7}$ M) may be due to contamination with traces of mitochondrial membranes.

There is much evidence that metyrapone (or metyrapol) binds to P-450. In particular, it induces marked changes in the optical spectrum of purified P-450 (Sweat *et al.*, 1969b; Peterson *et al.*, 1971). Furthermore, the competition between metyrapone or metyrapol and CO, or isocyanide, for binding to P-450 suggests that metyrapone binds to the O_2 side of the P-450 heme (Imai and Sato, 1968, and this paper). Previously the binding of metyrapone to P-450 has been determined from spectrophotometric experiments, which normally take into account only the total concentration of added metyrapone. This obviously tends to lead to an underestimation of the metyrapone binding affinity, as shown by some values reported in the literature, for example, 1×10^{-7} M for the binding of metyrapone to adrenal cortex mitochondria (Williamson and O'Donnell, 1969; Wilson *et al.*, 1969). It is interesting to note that the much lower K_d value (2.3×10^{-9} M calculated from spectral interaction of metyrapone with P-450 purified from *Pseudomonas putida* (Peterson *et al.*, 1971) was derived after adequate corrections for the ratio of bound and free concentrations.

The amount of metyrapol bound to adrenal cortex mitochondria (0.4–0.6 nmol/mg of protein) is lower than their content in P-450 (1.1 nmol/mg of protein). It is unlikely that one metyrapol molecule reacts with two or several P-450 molecules because the association kinetics are clearly bimolecular, and a one to one stoichiometry has been demonstrated for the binding of metyrapone to purified cytochrome P-450 (Peterson *et al.*, 1971). A possible explanation lies on the occurrence of separate different types of P-450, one having a much higher affinity for metyrapol. Actually two distinct types of P-450 have already been isolated from beef adrenal cortex mitochondria (Jefcoate *et al.*, 1970; Young *et al.*, 1970), one specific for the 11β-hydroxylation of deoxycorticosterone, and another for the side-chain cleavage of cholesterol. Another clue for the occurrence of two types of P-450 in adrenal cortex mitochondria is the finding that the low-spin electron paramagnetic resonance signal of P-450 is diminished upon addition of deoxycorticosterone and on the contrary increased upon addition of 20α-hydroxycholesterol (Whysner *et al.*, 1969). Finally, the fact that the cholesterol side chain cleavage is much less sensitive to metyrapone than the deoxycorticosterone hydroxylation (Young and Hall, 1971; Wilson and Harding, 1973) is a further strong argument for concluding that the high-affinity binding site for metyrapol is located on the P-450 specific for the 11β-hydroxylation.

In beef adrenal cortex mitochondria, the steroid 11β-hydroxylation system and the P-450 are located in the inner mitochondrial membrane (Yago and Ichii, 1969; Satre *et al.*, 1969; Dodge *et al.*, 1970). In agreement with this localization, metyrapol binds specifically to the inner mitochondrial membrane. The subcellular binding specificity of metyrapol is further supported by experiments showing almost no binding of metyrapol to adrenal cortex microsomes. The ferricyanide method applied to adrenal cortex mitochondria has allowed us to locate the 11β-hydroxylation system on the inner side of the inner membrane, and it follows that metyrapol can be considered as able to penetrate the inner membranes of adrenal cortex mitochondria.

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