Studies on Adrenal Cortical Cytochrome P-450. II. Effects of Inhibitors of 11β Hydroxylation on Its Optical and Magnetic Properties*

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ABSTRACT: Since many substrates of cytochrome P-450 catalyzed hydroxylations produce changes in the optical properties of this cytochrome, the effects of several inhibitors of steroid hydroxylation have been examined. All are competitive inhibitors of 11β hydroxylation occurring in adrenal cortex mitochondria. Each was found to produce similar, marked changes in the difference spectrum of these mitochondria, very dissimilar to the optical change produced by the steroid substrate. The concentration dependence of this spectral change is similar to the concentration dependence of the inhibition of hydroxylation. Both inhibitors tested also altered the electron paramagnetic reasonance spectrum of typically low-spin cytochrome P-450. This effect in each case consists of a shift of the low-field signal (g = 2.42) to even lower fields (g = 2.47). It appears that each inhibitor interacts with adrenal cytochrome P-450 at or near the heme producing an altered ligand field. This results in the observed effects on the catalytic and optical properties of this cytochrome.

The inhibitor potency is apparently a result of the affinity of each inhibitor for the same (or similar) site on the cytochrome.

A number of studies have described difference spectra induced by substrates of mixed-function oxidases in liver microsomes (Remmer *et al.*, 1966; Imai and Sato, 1966, 1967; Schenkman *et al.*, 1967), adrenal microsomes (Narasimhulu *et al.*, 1965), and adrenal mitochondria (Cooper *et al.*, 1965; Harding *et al.*, 1968; Oldham *et al.*, 1968). In general, two types of spectral responses have been observed; one is characterized by a 420-mμ trough and a peak at about 385 mμ and is produced by adding barbiturates to liver microsomes (Remmer *et al.*, 1966), 17-hydroxyprogesterone[1] to adrenal microsomes (Narasimhulu *et al.*, 1965), and 11-deoxycorticosterone to sonicates of adrenocortical mitochondria (Cooper *et al.*, 1965): the second has a peak at 420–430 mμ and a trough at about 390 mμ and can be produced by adding aniline or nicotinamide to liver microsomes (Remmer *et al.*, 1966; Imai and Sato, 1966, 1967; Schenkman *et al.*, 1967).

We have confirmed and extended these observations in intact adrenal mitochondria and have suggested that the spectrum induced by hydroxylatable steroid represents the oxidized-minus-reduced difference spectrum of cytochrome P-450 (450) (Oldham *et al.*, 1968). However, recent studies indicate that a redox change in P-450 (induced by steroid substrate) cannot readily explain all aspects of the phenomenon. One can still obtain the typical 11-deoxycortisol-induced spectrum using submitochondrial particles apparently containing no reduced P-450 since the untreated particles do not develop a 450-mμ peak when equilibrated with carbon monoxide (Whysner and Harding, 1968).

Alternative explanations have been offered. Narasimhulu *et al.* (1965) suggest that the steroid substrate converts a pigment which is not readily oxidized into one which is easily oxidized. They interpret their steroid-induced difference spectrum, then, in much the same way as do Oldham *et al.* (1968); that is, as an oxidized-minus-reduced spectrum of some pigment in the hydroxylating system. Remmer *et al.*, (1966) and Cammer *et al.* (1966) have concluded from optical and electron paramagnetic reasonance spectra of liver microsomes as well as of adrenocortical tissue that the substrate interacts either with the heme of P-450, thus modifying the ligand field, or directly with the iron by replacing or altering one of the ligands. Imai and Sato (1967) suggest that the substrate interacts with a specific site on the protein moiety of P-450 inducing a conformational change in the cytochrome which secondarily alters the ligand field. Since these suggestions are not all mutually exclusive, it has been difficult to get data uniquely supporting or rejecting any one of these hypotheses. Support for the view that cytochrome P-450 is the pigment actually responsible for these peculiar spectral changes has been presented and summarized by Imai and Sato (1967) and by Oldham *et al.* (1968).

Observations indicating that several nonsteroidal substances produce similar spectral changes in adrenocortical mitochondria are presented in this report. Each of these substances is known to be an inhibitor of steroid 11β hydroxylation and these studies suggest that this inhibition parallels the effects of the inhibitor on the spectral properties of the mitochondria. The data indicate that inhibitors specific for

* From the Departments of Medicine and Biochemistry, University of Southern California School of Medicine, Los Angeles, California. *Received February 11, 1969.* Supported in part by U. S. Public Health Service Grant CA 07057, by American Cancer Society Grants P-294 and PRS-11, and by U. S. Public Health Service Research Development Award 1-K3-GM-5532 (B. W. H).

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[1] Trivial names used are: 17-hydroxyprogesterone, 4-pregnene-3,20-dione-17α-ol; 11-deoxycorticosterone, 4-pregnene-3,20-dione-21-ol; 11-deoxycortisol, 4-pregnene-3,20-dione-17α,21-diol; cortisol, 4-pregnene-3,20-dione-11β,17α,21-triol; androstenedione, 4-androsten-3,17-dione; metyrapone, 2-methyl-1,2-bis(3-pyridyl)-1-propanone, amphenone, 3,3-bis(p-aminophenyl)-2-butanone; RS-3504, (1-*O*-nitro-p-tolyl)imidazole.

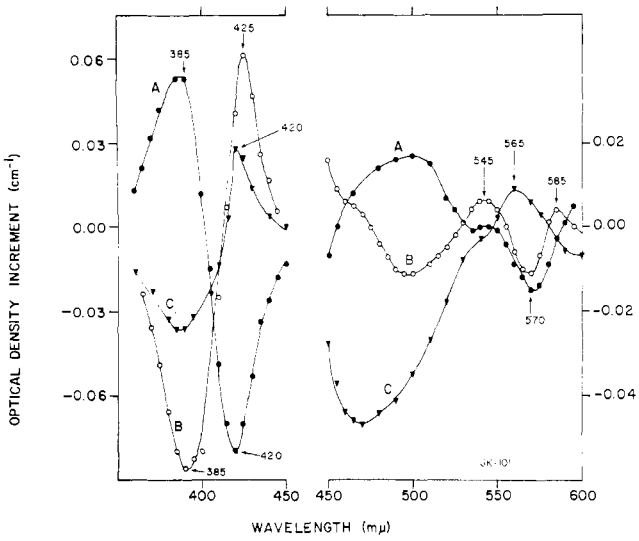

FIGURE 1: Effect of malate, 11-deoxycortisol, and metyrapone on difference spectra of adrenal cortical mitochondria. Each study was performed in 0.125–0.146 M sodium phosphate buffer (pH 7.4) and mitochondria equivalent to 27.5 mg of protein for the 700–450-mμ region and 13.7 mg of protein for the 360–450-mμ region. In addition, spectrum A,A and spectrum B,B contained 20 μM 11-deoxycortisol and 100 μM metyrapone, respectively, in the sample cuvet. Spectrum C,C contained 10 mM succinate, 6.0 mM KCN, 50 μg of antimycin A in both the sample and reference cuvet, and 5 mM malate in the sample cuvet.

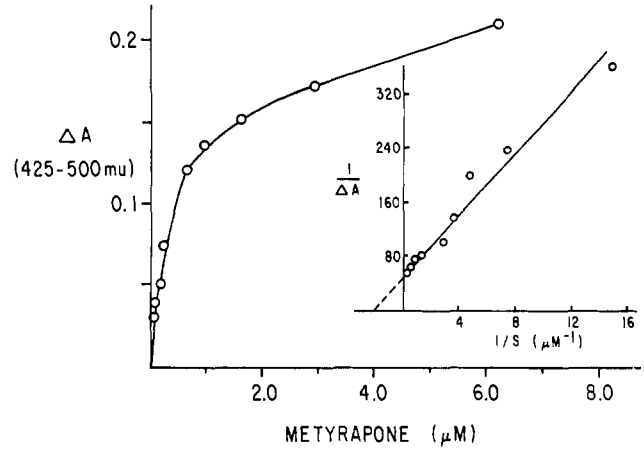

FIGURE 2: Effect of increasing concentrations of metyrapone on the absorbancy at 425 mμ. Mitochondria equivalent to 2.6 mg of N were suspended in 6.0 ml of phosphate buffer (pH 7.2) and divided equally into two cuvets. Difference spectra were obtained following successive additions of metyrapone to the sample cuvet and water to the reference. The absorbancy difference between 500 and 425 mμ is plotted against the metyrapone concentration (corrected for dilution). The inset gives the same data in double-reciprocal form. The estimated dissociation constant, K_s, is about 0.4 μM.

11 hydroxylation produce their effect by interacting with cytochrome P-450. This interaction apparently alters the ligand field of this cytochrome resulting in a form whose catalytic activity is hindered.

Materials and Methods

Beef adrenal glands were collected, processed, and the mitochondrial fraction was isolated by differential centrifugation as described previously (Oldham *et al.*, 1968). Optical spectra, electron paramagnetic resonance studies, assays of steroid 11β hydroxylation, and kinetic studies with the Aminco–Chance dual-wavelength spectrophotometer were all done using methods previously described (Oldham *et al.*, 1968).

Specific conditions for each experiment are described in the legends to the appropriate figures.

Results

As reported previously, steroid substrates of 11β hydroxylation induce a characteristic difference spectrum when added to bovine adrenocortical mitochondria (Figure 1, curve A,-A). The structural requirements for inducing this spectrum are reasonably specific since a number of related steroids (including the 11β-hydroxylated products) produced no such difference spectra (Oldham *et al.*, 1968).

The effect of a known inhibitor of 11β hydroxylation, metyrapone, on the spectra of these adrenocortical mitochondria is shown in Figure 1 (curve B,B). This spectrum was obtained by adding metyrapone to the sample cuvet containing untreated aerobic mitochondria read against untreated aerobic mitochondria. The similarity of this spectrum to the spectrum produced by malate (curve C,C) is obvious. However, characteristic differences are seen: all peaks are shifted to longer wavelengths and the β band (545 mμ) is more intense than the α band (595 mμ). These differences are consistently seen and are also demonstrated when metyrapone is added to the sample cuvet already containing malate (*e.g.*, malate + antimycin A + succinate + air) as reported by Harding *et al.* (1968). The difference spectrum induced by metyrapone is associated with competitive inhibition of steroid 11β-hydroxylase with an apparent K_i of about 0.2 μM (Harding *et al.*, 1968; Williamson and O'Donnell, 1967). The effect of increasing concentrations of metyrapone on the magnitude of the Soret peak is shown in Figure 2. The inset gives a double-reciprocal plot of the data, indicating an approximate spectral dissociation constant, K_s, of 0.4 μM which is similar to the K_i of this substance.

A kinetic study of the effects of metyrapone on 11β hydroxylation and spectral changes in mitochondria is shown in Figure 3. The control run is illustrated by the upper set of traces which indicate the time course of the change in absorbancy difference at 420–405 mμ (A) correlated with oxygen utilization (A′) and the appearance of 11β-hydroxylated product (cortisol) (A″) in the same cuvet. Succinate produces no significant change in the rate of oxygen uptake in these antimycin A treated mitochondria although a downward deflection of the absorbancy tracing does occur due to reduction of cytochrome *b* which results in an increase in the 420–405-mμ difference. Malate then causes a greater increase in absorbancy at 420 mμ perhaps indicating additional reduction of cytochrome *b* but undoubtedly reflecting the appearance of the 420-mμ peak shown in Figure 1 (curve C). Addition of 11-deoxycortisol produces a rapid and marked upward deflection indicating a decrease in absorbancy at 420 mμ relative to 405 as previously shown by curve A in Figure 1. The rate of cortisol formation determined from repeated small aliquots of the cuvet contents is shown by the open circles. 11β

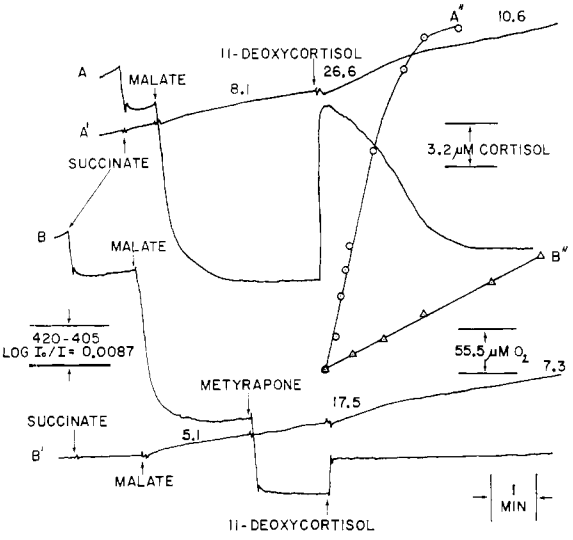

FIGURE 3: Correlation of spectral changes and oxygen uptake with 11β hydroxylation. Mitochondria equivalent to 1.3 mg of N were suspended in phosphate buffer containing 0.03% (w/v) bovine serum albumin, antimycin A (10 μg), and cyanide (1.5 mM). The absorbancy difference between 420 and 405 mμ was monitored with the dual-wavelength machine together with oxygen tension in the same cuvet. An increase in the absorbancy difference is shown by a downward deflection. Additions were made as indicated giving final concentrations of: succinate (3 mM), malate (3 mM), [$^{14}C_4$]11-deoxycortisol (32 μM). The numbers above the oxygen traces indicate the rate of oxygen uptake, micromolar per minute. The rate of hydroxylation, as determined from multiple small aliquots from the same cuvet, is indicated by open circles. The lower pair of traces illustrate the effects of metyrapone (0.8 μM) on these changes, with hydroxylation indicated by the triangles.

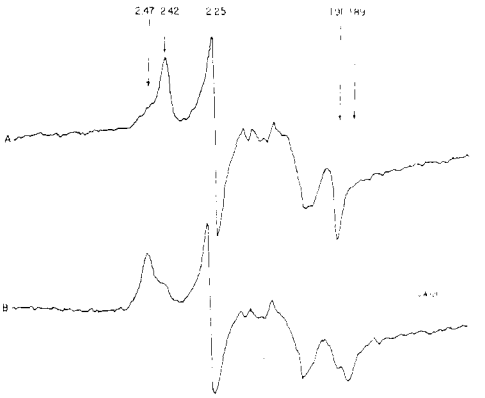

FIGURE 4: The effect of metyrapone on the electron spin resonance spectrum of submitochondrial particles. The concentration of particles was 35 mg of protein/ml. A, particles with no additions; B, particles with 100 μM metyrapone. Electron paramagnetic resonance parameters were as follows: power, 200 mW; temperature $-170°$; modulation frequency, 100 kHz, modulation amplitude, 20 gauss, scan range, 2000 gauss; scan time, 8 min. Measurements were made using the Varian E-3 spectrometer with a variable-temperature accessory.

Hydroxylation is linear while the absorbancy difference is maximal and in a steady state, lasting about 1 min. The rate of hydroxylation decreases as the absorbancy difference decreases and finally stops as the absorbancy trace returns toward the presteroid level and a new steady state. The lower set of traces illustrates the effect of metyrapone on this cyclic spectral change associated with 11β hydroxylation. As shown by the triangles, hydroxylation activity is inhibited approximately 80% by 0.8 μM metyrapone. The absorbancy changes producing the differences indicated by the tracing are complex but a moderate downward deflection on adding the inhibitor is noted (line B). This is expected since the 420-mμ peak known to be present (due to malate) is intensified by the appearance of the 425-mμ band, as shown in Figure 1. The result is an increased absorbancy at 420 relative to 405 mμ and a downward deflection of the tracing. The sharp upward deflection seen in the upper tracing on adding 11-deoxycortisol is markedly damped in the metyrapone-treated sample. Difference spectra obtained after the addition of 11-deoxycortisol to metyrapone-treated mitochondria confirm the above interpretation, showing a progressive loss of the 420-mμ trough normally induced by this steroid as the concentration of metyrapone is increased.

The effect of metyrapone on the electron paramagnetic resonance spectrum of adrenocortical submitochondria particles is shown in Figure 4. The upper tracing is the spectrum at 77°K of aerobic particles treated with 10 μg of antimycin A. Antimycin A helps prevent anerobiosis in these very concentrated preparations. Signals at g = 2.42, 2.25, and 1.91 typical of oxidized, low-spin P-450 are apparent. The spectrum obtained by adding metyrapone (100 μM) to aerobic mitochondria is shown by tracing B. A significant shift in the 2.42 signal to 2.47 is clearly shown with a just discernible change in the position of the 1.91 signal to 1.89.

The spectral effects of amphenone (3,3-bis(p-aminophenyl)-2-butanone), another inhibitor of corticoid production, are shown in Figure 5 by the open circles. The bands observed at 427, 548, and 585 mμ with a trough at about 390 mμ are quite similar to those induced by metyrapone. Figure 6 demonstrates the effect of increasing concentrations of amphenone on the magnitude of the spectral change. The inset presents the data in reciprocal form and indicates a K_s of about 200 μM. This value is somewhat less than the apparent K_i for inhibition of 11β hydroxylation (see below) but is similar to its K_i for the inhibition of cholesterol side-chain cleavage in this tissue (Kibelstis and Fergusson, 1964).

The kinetics of the effect of amphenone on the spectral properties of these mitochondria are shown in Figure 7. The abbreviated lower tracing illustrates the stepwise changes in the absorbancy difference of 430–420 mμ monitored by the dual-wavelength spectrophotometer in the absence of amphenone. Antimycin A has already been added to the cuvette and the initial part of the tracing indicates the resultant steady state. Changes produced by the successive additions of succinate, malate, and 11-deoxycortisol are similar to those described in Figure 3. The upper tracing illustrates a similar train of events but addition of amphenone after succinate results in a large, rapid increase in absorbancy at 430 mμ, as illustrated by the previous difference spectrum (Figure 5). Addition of malate now results in a small, slow, perhaps insignificant downward deflection apparently reflecting the inability of malate to further alter P-450. Hydroxylatable steroid can still induce the 420-mμ trough as indicated by the upward deflection initiated by 11-deoxycortisol. Although this upward deflection is less than induced by steroid in the control run, the duration of the trough (*i.e.*, the time required

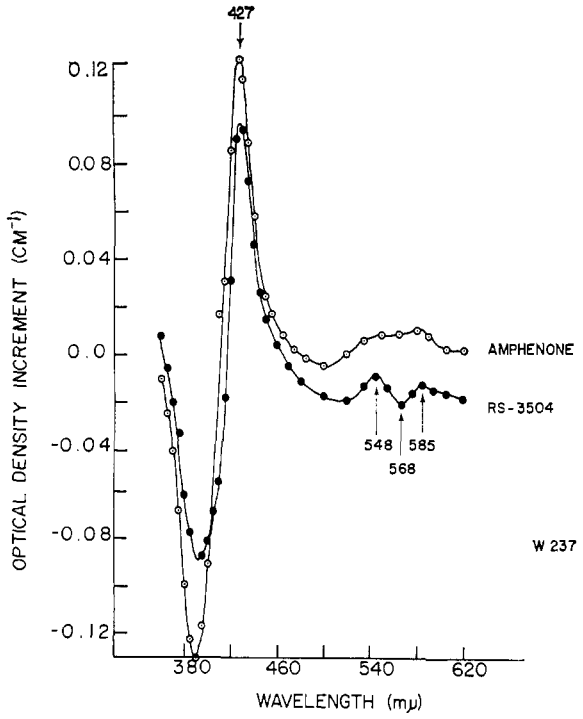

FIGURE 5: Difference spectra produced by RS-3504 (solid circles) and by amphenone (open circles). In each case mitochondria equivalent to 4.9 mg of N were suspended in 6.0 ml of phosphate buffer and this volume was then distributed equally into two cuvets. After obtaining a base line of equal light absorbancy, RS-3504 was added to the sample cuvet in a final concentration of 32 μM and an equal volume of ethanol to the reference cuvet. The curve indicated by the solid circles was obtained by subtracting the base line as described previously (Oldham *et al.*, 1968). The amphenone curve (open circles) was obtained similarly using a final concentration of inhibitor of 830 μM in a separate run.

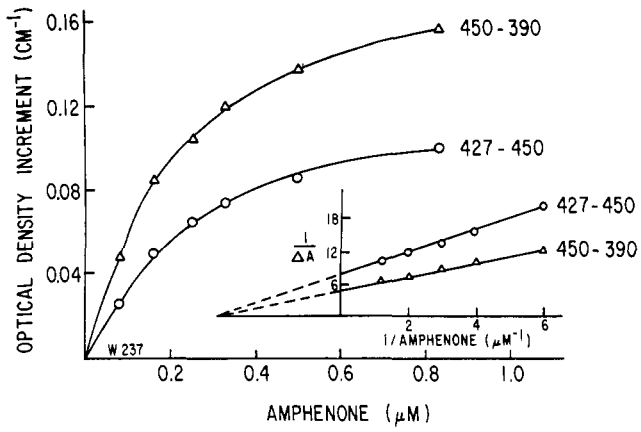

FIGURE 6: Titration of the spectral effect of amphenone. The study was done as described in Figure 2. Tissue equivalent to 4.9 mg of N was suspended in 6.0 ml of phosphate buffer and then divided equally. The two curves represent the changes in the Soret maximum (open circles, 427–450 mμ) and the 390-mμ trough (triangles). The inset gives the same data in double-reciprocal form and indicates an approximate K_s of about 260 μM for both trough and peak.

for the slow return toward the presteroid level, indicative of disappearance of 11-deoxycortisol) is about the same. Additional incubation studies indicate that amphenone is a relatively weak inhibitor of 11β hydroxylation showing competitive kinetics with a K_i of about 0.5 mM (Harding *et al.*, 1968).

A newly discovered inhibitor of 11β hydroxylation, RS-3504 (1-*O*-nitro-*p*-tolyl)imidazole, is reported to be competitive in nature but somewhat less potent than metyapone (R. I. Dorfman and D. Sharma, 1967, personal communication). The difference spectrum induced by this substance is shown in Figure 5 by the solid circles and is seen to be virtually identical with that produced by metyrapone (Figure 1, curve B). This spectrum is produced whether or not reducing conditions are present and, like the metyrapone-produced spectrum, it is not altered by the subsequent addition of 11-deoxycortisol. A titration of the Soret spectral change produced by RS-3504 is shown in Figure 8 with the double-reciprocal plot given in the inset. The estimated dissociation constant is 5–6 μM which is about tenfold greater than that found for metyrapone. This value is approximately the same as the K_i for inhibition of 11β hydroxylation.

The kinetics of the effect of RS-3504 are shown in Figure 9 in which the wavelength pair 425–420 mμ is monitored. The upper pair of lines (A,A′) show the previously described sequence of events occurring in the absence of inhibitor. The line drawn through the circles (A′) starting at the time of addition of 11-deoxycortisol indicates the formation of 11β-hydroxylated product (cortisol). The rate of hydroxylation is linear throughout the 3-min period studied with approximately 60% of the substrate hydroxylated during this time. Lines (B,B′) (C,C′), and (D,D′) illustrate the effect of increasing RS-3504 concentrations on hydroxylation, and the absorbancy difference. The sequence of events is the same until the inhibitor is added after succinate. At the highest concentration of inhibitor used (4.4 μM) a rapid, large increase in absorbancy at 425 relative to 420 mμ occurs (line D,D′) reflecting the absorbancy difference shown in Figure 5. Malate causes a small deflection in the opposite direction but addition of 11-deoxycortisol produces virtually no change in the spectral tracing and oxygen uptake (not shown here) is only slightly affected. These differences from the control (A,A′) are reflected by a retarded rate of hydroxylation maximally shown by line D′. This rate is about 30% of the uninhibited rate. Similar studies using lower concentrations of RS-3504 (0.9 μM, lines B,B′; 2.2 μM, lines C,C′) show qualitatively similar changes with essentially no deviation from the control run occurring at the lowest concentration. The apparent K_i for RS-3504 calculated from these and similar data is approximately 3.5 μM.

The effect of RS-3504 on the electron paramagnetic resonance spectrum of these mitochondria is shown in Figure 10. The upper trace is the control without inhibitor. Like metyrapone, this inhibitor also produces a significant shift in the $g = 2.42$ signal to about $g = 2.52$ (shown in the lower tracing) without appreciably affecting the intensity or the position of the 2.26 signal.

Discussion

Previous reports have indicated that in microsomes some agents which are not substrates for mixed-function oxidases can produce spectral changes similar to those induced by sub-

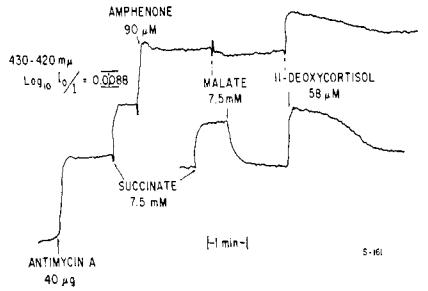

FIGURE 7: Kinetic effects of amphenone on the spectral properties of adrenal mitochondria. Mitochondria equivalent to 0.75 mg of N/ml suspended in phosphate buffer (pH 7.2). The difference in absorbancy between 430 and 420 mμ was monitored with the dual-wavelength spectrophotometer and an increase in this difference is indicated by an upward deflection. Additions were made as shown on the figure.

strates. Narasimhulu *et al.* (1965) observed that androstenedione produced a 420-mμ trough in adrenocrotical microsomes even though it cannot be a substrate for the 21-hydroxylase system present. Remmer *et al.* (1966) observed two kinds of spectral responses on adding two different inhibitors of drug oxidation to liver microsomes. More recently a variety of aliphatic alcohols have been shown to produce similar spectral changes in liver microsomes (Imai and Sato, 1967). While the significance of the kind of spectrum produced by any agent is not clear, Oldham *et al.* (1968) have suggested that the difference spectrum induced in adrenocortical mitochrondria by 11-deoxycortisol (420-, 535-, and 570-mμ troughs) is due to a redox change in cytochrome P-450 resulting in an oxidized-minus-reduced spectrum of this pigment. This view suggests that the second kind of spectral alteration (peak at 420–430 mμ) represents the reduced-minus-oxidized spectrum of the cytochrome or at least of the pigments in the hydroxylating pathway. This explanation accounts for the enhancement of the difference spectrum by malate ("reducing conditions") and 11-deoxycortisol ("oxidizing conditions") in opposite cuvets (Oldham *et al.*, 1968), and the decrease in the magnitude (Schenkman *et al.*, 1967) of the aniline-induced spectrum caused by adding reducing equivalents to both cuvets. However, this suggestion is not compatible with the observation that 11-deoxycortisol produces its characteristic difference spectrum in submitochondrial particles which are already entirely oxidized (Whysner and Harding, 1968). In addition, studies by Horie *et al.* (1966) on the "absolute" spectra of adrenal mitochondrial

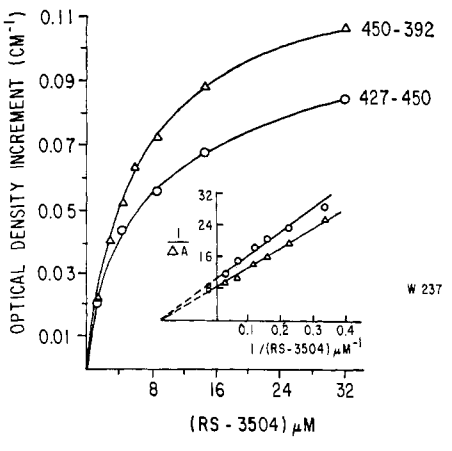

FIGURE 8: Titration of the spectral effect of RS-3504. The study was done exactly as described in Figure 6 using successive additions of RS-3504. Again the open circles indicate the appearance of the Soret peak (427–450 mμ) and the triangles represent the changes in the trough (450–392 mμ). The inset indicates an approximate K_s of 6 μM for both the peak and trough.

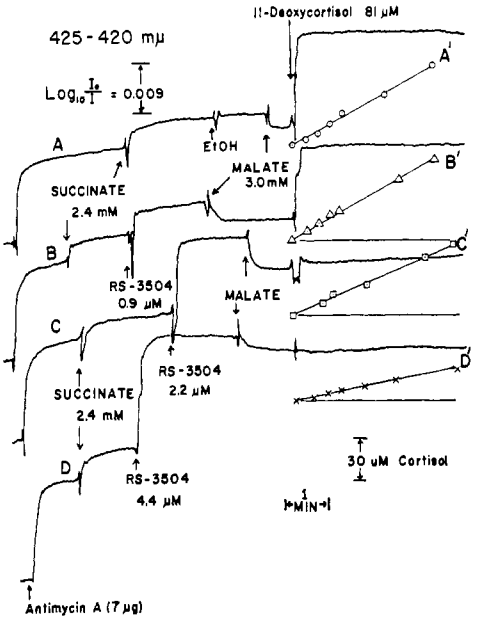

FIGURE 9: Effect of RS-3504 on steroid induced spectral changes and 11β hydroxylation. Mitochondria equal to 1.9 mg of N were suspended in 3.2 ml of phosphate buffer and the absorbancy difference between 425 and 420 mμ was monitored in the dual-wavelength machine and an upward deflection indicates an increase in this difference. Oxygen tension recordings have been omitted. Additions were made as indicated and the rates of hydroxylation are given by the straight lines starting at the time of addition of [$^{14}C_4$]11-deoxycortisol. Tracing A is the control and subsequent tracings illustrate the effects of increasing concentrations of RS-3504.

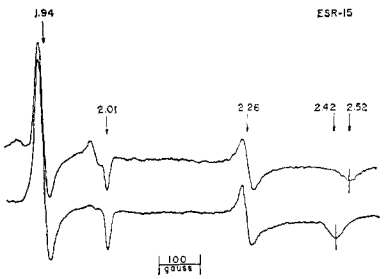

FIGURE 10: Effect of RS-3504 on the electron paramagnetic resonance spectrum of adrenal mitochondria. First derivative spectra were obtained at 77°K on a Varian Model 4502 spectrometer. Mitochondria equivalent to 4.4 mg of N were suspended in phosphate buffer and treated with malate (8 mM) and antimycin A (10 μg). After aerating, ethanol (lower tracing) or RS-3504 (upper tracing) was added and the sample was frozen. Scan speed was 200 gauss/min with power of 100 mw. The final concentration of the inhibitor was 100 μM.

particles and by Nishibayashi and Sato (1967) on microsomal P-450 are not consistent with the redox hypothesis.

The difference spectrum produced by metyrapone differs significantly from the malate-produced difference spectrum and from the inverse of the 11-deoxycortisol-induced difference spectrum. These changes are a shift to longer wavelengths and an intensification of the β band and these occur whether or not the typical malate-induced spectrum is present. Together with the unequivocal shift in the low-field electron paramagnetic resonance signal of oxidized cytochrome P-450 from $g = 2.42$ to $g = 2.47$ caused by metyrapone, these data suggest that this agent does not produce a simple redox change in P-450, but rather that it either distorts the ligand field of cytochrome P-450 or actually replaces one of its ligands. This action of metyrapone may also be consistent with an acceleration of oxygen uptake and a decrease in levels of reduced pyridine nucleotides which have been reported using adrenal mitochondria (Harding *et al.*, 1968; Williamson and O'Donnell, 1967; Sanzari and Peron, 1966). A leak of electrons to oxygen from the metyrapone-bound P-450 (or some component of the hydroxylating pathway prior to P-450) could account for these observations and still permit a fairly specific inhibition of 11β hydroxylation.

The findings obtained using RS-3504 are very similar to those just discussed for metyrapone. Although it is less potent as an inhibitor of 11β hydroxylation, its effects on the difference spectrum and electron paramagnetic resonance spectrum are virtually identical. While the effect of this inhibitor on oxygen utilization and the level of reduced pyridine nucleotides is not known, one might predict effects similar to those observed with metyrapone.

Amphenone behaves somewhat differently even though its structure is quite similar to that of metyrapone. Amphenone is less potent by two to three orders of magnitude in inhibiting hydroxylation of 11-deoxycortisol and also appears to be considerably less specific for this step (Rosenfeld and Bascom, 1956). The difference spectrum produced by amphenone is also different in that it is more readily reversed by 11-deoxycortisol, perhaps indicating only a lower affinity of this inhibitor for the hydroxylating system. The site of this interaction is unknown and electron paramagnetic resonance data is lacking.

Recent studies by Hildebrandt *et al.* (1968) indicating that P-450 in liver microsomes exist in two different spin states suggest that some of the spectral alterations described here could be due to spin state changes. For example, 11-deoxycortisol might cause a low-spin into high-spin conversion while the inhibitors, such as metyrapone and RS-3504 might produce the high-spin into low-spin conversion. Additional electron paramagnetic resonance studies of adrenocortical mitochondrial P-450 have not yet provided support for this view. Hydroxylatable substrates produce only small alterations (Harding *et al.*, 1968; Oldham *et al.*, 1968; Cammer *et al.*, 1966) in the low-spin signals of P-450 with no evidence for the generation of a high-spin hemoprotein signal ($g = 6.0$). The concentration-dependent shifts of the $g = 2.42$ signal to lower fields produced by metyrapone and RS-3504 are not accompanied by changes in the high-spin signal. It is recognized that electron paramagnetic resonance spectra obtained at liquid nitrogen temperature may not be correlated with absorption spectroscopy done at room temperature since Yonetani *et al.* (1966) have shown that the spin state of one hemoprotein (cytochrome *c* peroxidase) is temperature dependent.

Although it is not clear just what changes in P-450 are produced by metyrapone and RS-3504, it appears that each does interact with this cytochrome. This interaction is manifested in each case by distinct changes in the optical and electron paramagnetic resonance properties of P-450 suggesting that each inhibitor similarly modifies the ligand field of P-450, perhaps by replacing a ligand. The result is a form of P-450 which apparently cannot interact in the usual way with the substrate to be hydroxylated (11-deoxycortisol). The competitive nature of the resulting inhibition of 11β hydroxylation suggests that both inhibitors and the steroid substrate either bind at a common site or at interacting sites. Although data on the action of amphenone are less complete, the spectral and competitive inhibitory effects of this agent are similar to those of metyrapone and RS-3504. It appears that all three inhibitors function in a similar if not identical way and that the marked differences in potency (more than three orders of magnitude) are a reflection of their greatly different affinities for cytochrome P-450 indicated by their respective spectral dissociation constants.

Acknowledgment

We thank Mrs. Gertrude Kazmi and Mrs. Remedios Limon for their technical assistance.

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Microbial Sulfolipids. III. The Disulfate of (+)-1,14-Docosanediol in *Ochromonas danica**

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ABSTRACT: A new sulfolipid, the disulfate of (+)-1,14-docosanediol, was isolated from the phytoflagellate, *Ochromonas danica*. It was characterized by synthesis of (DL)-1,14-docosanediol and its disulfate and by comparison of the synthetic materials with the natural materials. In order to determine the orientation of the natural secondary sulfate, it was necessary to explore the mechanism of solvolysis of sulfate esters in dioxane.

It was found that solvolysis of secondary sulfates to alcohols in dioxane occurs with retention of configuration around the C–O bond of the alcohol. Comparison of the natural (+)-1,14-docosanediol rotation with the rotations of hydroxy acids of known absolute configuration suggest the natural sulfolipid is 1-(*S*)-14-docosanediol 1,14-disulfate. With one exception all other natural hydroxy fatty acids (where the hydroxyl is isolated and beyond the β position) have the *R* configuration. Chlorodocosanediols are obtained from the solvolyzed sulfatides.

Phospholipids have been found in all organisms in which they have been sought to date. Sulfolipids have been less sought than phospholipids but it now appears they are ubiquitous. The retarded development of sulfolipid research has been due to the poorer analytical methods for sulfate, whereas phosphate is conveniently assayed by molybdate. The recent availability of sulfur-35 has permitted the identification of a variety of sulfolipids including the sulfonolipid[1] of the chloroplast (Daniel *et al.*, 1961), the sulfatide of *Halobacterium cutirubrum* (Kates, 1967), and the sulfatides described in this series.

Perhaps the most surprising aspect of the sulfatide, herein described, is the fact that the polar groups, always charged because of the extremely low pK_a of sulfates, are essentially at *both ends* of the molecule. To our knowledge, no similar lipid has been found in living tissue although several compounds with polar groups at both ends of the molecule have been found to be *excreted* by yeasts (Stodola *et al.*, 1967; Gorin *et al.*, 1961).

In order to characterize the sulfatide, it was necessary to determine the configuration of the carbon at position 14. It was not possible to obtain the rotation of the secondary sulfate directly because of its poor solubility and its tenacity for trace impurities throughout its isolation and purification. Acid hydrolysis was expected to produce some racemization and a poor yield of diol. Solvolysis in dioxane appeared to be a mild, rapid, and highly specific technique (McKenna and Norymberski, 1957; Burstein and Leiberman, 1958). Unfortunately the effect of solvolysis of secondary alkyl sulfates upon the configruration of the C–O bond was not known. Experiments with optically active 2-octanol 2-sulfate demonstrated that solvolysis occurred with retention of configuration.

This procedure, together with the unambiguous synthesis of 1,14-docosanediol 1,14-disulfate, has permitted a complete characterization of the sulfatide in *Ochromonas danica*. This structure had earlier been proposed for the sulfatide (Mayers and Haines, 1967).

Experimental Procedure

Materials. All reagents were analytical grade. Solvents were distilled before use. Dioxane was distilled from lithium aluminum hydride and used immediately. 12-Bromolauric acid (Sapon Laboratories) was recrystallized from heptane and then from methylene chloride (mp 51.5–52.5°). Diethyl malonate, dihydropyran (City Chemical Co.), and nonanoic acid were redistilled before use and checked for purity by gas chromatography. (−)-2-Octanol (Aldrich Chemicals), $[\alpha]_D^{23}$ −10.05° (*c* 8.6, ethanol), was checked for purity by gas chromatography.

Isolation of the Sulfatide. O. danica was cultured on defined medium as described earlier (Haines, 1965). The procedure for isolation of the sulfatide has already been described (Mayers and Haines, 1967; Haines, 1965). A brief summary of this procedure follows.

Cells were collected by centrifugation and extracted three

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[1] The term *sulfolipid* in this paper means a sulfur-containing lipid; *sulfatide* refers to a lipoid sulfate ester; *sulfonolipid* is a sulfolipid in which the sulfur occurs as a sulfonic acid.