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TWO ADRENAL GLAND CYTOCHROME P-450 ENTITIES EVIDENCED BY METYRAPONE-INDUCED SPECTRA

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SUMMARY

1. Marked diminution in the metyrapone-induced 425-nm difference spectrum occurs when 17-hydroxyprogesterone is added to microsomal preparations, but only a low magnitude response is observed when this steroid is introduced into mitochondrial fractions exhibiting metyrapone spectra. Deoxycorticosterone induces spectra in both acellular fractions, but does not influence the magnitude of metyrapone spectrum.

2. Diminution of the microsomal metyrapone absorption peak after addition of 17-hydroxyprogesterone, appears to be primarily due to summation of the 420-nm absorption minimum induced by 17-hydroxyprogesterone and the 425-nm absorption maximum induced with metyrapone.

3. Metyrapone cancels deoxycorticosterone-induced spectra occurring either in microsomes or mitochondria and attains a magnitude identical to that attained in the absence of deoxycorticosterone.

4. Addition of metyrapone to microsomal preparations containing deoxycorticosterone results in the same difference spectrum as that manifest by metyrapone alone, suggesting complete displacement of the spectral inducing elements of deoxycorticosterone in preference to those of metyrapone. Such a manifestation, however, does not occur in respect to 17-hydroxyprogesterone. The spectral shift which occurs when metyrapone is added to preparations containing 17-hydroxyprogesterone is distinct from metyrapone alone. When metyrapone is added to the reference cuvette, the sample cuvette reveals the 17-hydroxyprogesterone spectrum; when 17-hydroxyprogesterone is added to the reference cuvette the metyrapone spectrum is revealed indicating independence of the two substrates in inducing their respective difference spectra.

INTRODUCTION

11-Deoxy-21-hydroxysteroid-induced spectra in adrenal gland mitochondrial preparations and C-21 deoxysteroid-induced spectra in microsomal preparations have been reported by several investigators[1–4]. These observations are generally considered,

although not established, to be associated with cytochrome P-450. Similarities of certain reactions in mitochondria and microsomes argue for a single species of cytochrome P-450, while other reactions are indicative of separate species. The common requirement of NADPH[5–7] and O_2 (refs. 8–11) and the manifestation of identical CO-induced spectra associated with 11β- and 21-hydroxylation is compatible with the former concept. Specificity of inhibitors and distinctive requirement of relatively large quantities of soluble adrenal flavoprotein and nonheme iron proteins for 11-hydroxylation in partially purified preparations, in contrast to the singular requirement of NADPH in microsomal 21-hydroxylating preparations, suggest different species of cytochrome P-450.

In the present investigation, characteristics of the steroid 11β-hydroxylation inhibitor, metyrapone, are employed to demonstrate the existence of two distinct cytochrome P-450 systems in adrenal gland cortex particles. It is observed that metyrapone induces identical qualitative spectra in mitochondria and microsomes prepared by conventional centrifugation methods. However, a distinction exists in the response of the two preparations upon addition of 17-hydroxyprogesterone. A marked diminution occurs in the spectrum of microsomal preparations in contrast to only a minor change in that of the mitochondrial particles. The difference is attributed to the relative proportions of the steroid 11- and steroid 21-hydroxylating systems present in the two preparations. The 11β-hydroxylation system (on the assumption of a mitochondrial specificity) contaminates the microsomal elements to a much greater extent than contamination of mitochondria with the C-21-hydroxylating system. As the steroid-induced spectra in mitochondria are demonstrable in preparations free of reductase components[4], it is concluded that spectra are associated either directly with cytochrome P-450 or a closely related companion pigment.

It is well documented that adrenal microsomal fractions, as prepared by contemporary techniques, show mitochondrial contamination[12–14]. All differential centrifugation methods investigated in this laboratory[15,16] including those described by SHARMA AND DORFMAN[17] and ESTABROOK *et al.*[12] have been found to resolve microsomal fractions which, when supplemented with cytochrome P-450 reductase, support a significant degree of steroid 11β-hydroxylation[15] and respond spectrophotometrically to deoxycorticosterone*, 17-hydroxyprogesterone and metyrapone. Failure to observe significant steroid 11β-hydroxylation in *in vitro* incubations of "back washed" microsomes in the past seems largely accounted for in the absence of mitochondrial cytochrome P-450 reductase which is removed** during processing of microsomal preparations. The present investigation takes advantage of this mitochondrial-like

* Although the specific steroids examined were not noted, COOPER *et al.*[17] reported that 21-hydroxylated steroids do not induce a spectra response at 420 nm in adrenal gland microsomes. Authors of the present paper, employing the same differential centrifugation method as these investigators, consistently observe a response with the 21-hydroxylated steroid, deoxycorticosterone. Further, the referred to authors did not report that 17-hydroxyprogesterone induces a response in mitochondria (a constant finding in this laboratory) similar to that in microsomes.

** It is not clear whether the steroid 11β-hydroxylation observed in adrenal gland microsomes is due to fragmented mitochondria or whether it represents an innate form of this enzymic system present in the endoplasmic reticulum. Irrespective of the source, however, it is apparent that sedimentation of microsomes from mitochondrial supernatant medium, together with any backwashing such as prescribed by several contemporary techniques, effectively leaches components of the soluble cytochrome P-450 reductase system from the insoluble particles, thus masking 11β-hydroxylation capacity unless refortified with these components.

activity in microsomal fractions to examine the respective natures of the steroid 11- and 21-hydroxylating systems*.

EXPERIMENTAL AND RESULTS

Samples of bovine adrenal gland microsomes, prepared according to the method of ESTABROOK *et al.*[12] were treated with 0.01 M metyrapone (Fig. 1), which was added in small increments to the sample cuvette with shaking until the spectrum was stab-

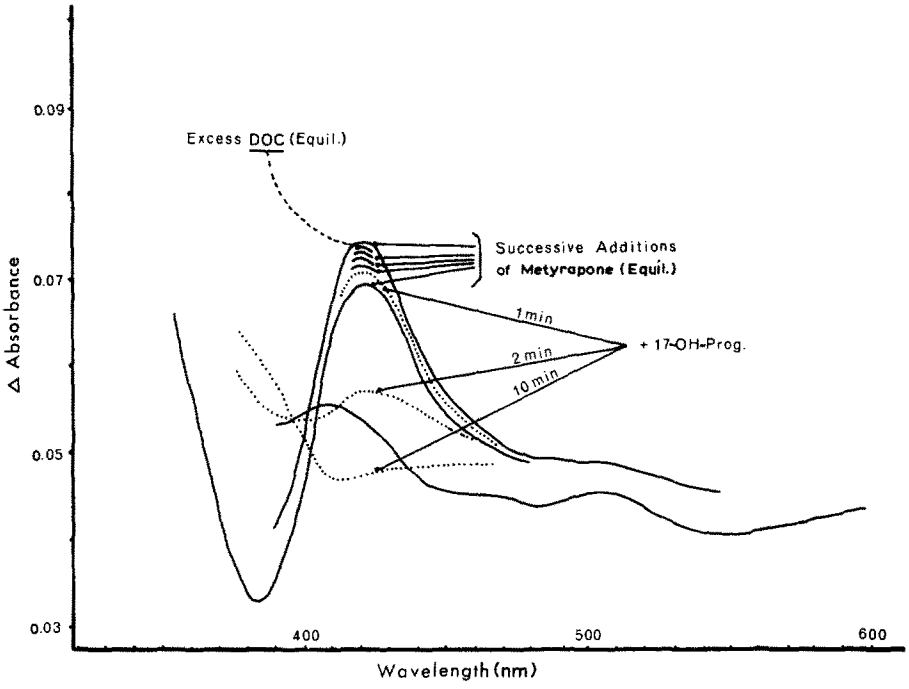

Fig. 1. Influence of deoxycorticosterone (DOC) and 17-hydroxyprogesterone on microsomal spectra induced by metyrapone. 3 ml of the microsome suspension (4.1 mg protein/ml) was placed in each of two cuvettes (3 ml) with 1-cm light pathway and the solid baseline, extending to the right in the drawing, recorded. 0.01 M metyrapone was added in increments of 50 μl to the sample cuvette over a period of 10 min after which interval the spectrum was stabilized. The maximum recording contained 200 nmoles. Several small crystals of deoxycorticosterone were added with agitation and monitored by the spectrophotometer (Cary 15) over a period of 10 additional minutes, during which time the spectrum stabilized as indicated by the recorded line second from the top in the figure. Crystals of 17-hydroxyprogesterone were finally added and the spectra indicated by the dotted lines recorded over a period of 10 min.

* The degree to which adrenal mitochondrial-like activity is present in microsomes is striking. On the basis of appropriate time and substrate concentrations (unpublished data), the capacity of microsomal preparations to carry out 11β-hydroxylation (after addition of the reductase) approaches 25% of that in mitochondrial preparations processed (either by pestle or blade homogenization) at differential speeds between $9000 \times g$ (20 min) and $12\,000 \times g$ (30 min). If the activity is due to a general fragmentation in these preparations, a simple calculation reveals that a 1:4 contamination would be required. On the other hand, if specific dissolution of light mitochondria, which have been found to exhibit a very high specific activity of 11β-hydroxylation, occurs, contamination on a protein basis would be of a much less degree. Dr. Melvin J. Bryson of this laboratory has demonstrated steroid 11β-hydroxylation specific activities of light mitochondria to approach 1800 nmoles conversion per mg protein per h.

ilized. Crystals of deoxycorticosterone were next added with shaking during which no change of spectrum was observed. Finally, a single addition of 17-hydroxyprogesterone crystals was added with a resulting progressive diminution of the 425-nm spectrum as indicated by the dotted lines of the figure. The time factor relates to the slow rate of solution of the 17-hydroxyprogesterone crystals and not to the rate of the steroid reaction responsible for the spectral change. Previous studies in this laboratory[4] have shown that the 425-nm absorption peak of metyrapone (WILSON *et al.*[18] have also reported an absorption spectrum for metyrapone in adrenal gland preparations) replaces the 419–420-nm trough induced by deoxycorticosterone and that the subsequent addition of deoxycorticosterone does not reverse the spectrum. This is again demonstrated in the present experiment. However, in contrast to the unresponsiveness of the metyrapone spectrum to deoxycorticosterone, a marked decrease in the 425-nm spectral peak is caused by 17-hydroxyprogesterone. This appears to be primarily due to superimposition of a 17-hydroxyprogesterone-induced trough at 420 nm which cancels the 425-nm peak. That the observations are not due to displacement of metyrapone is evidenced when metyrapone was added to a sample cuvette containing 17-hydroxyprogesterone exhibiting a spectrophotometric trough at 420 nm. The trough was displaced upward beyond the baseline with a positive 423–425-nm peak (Fig. 2). When metyrapone was added to the reference cuvette, the 17-hydroxy-

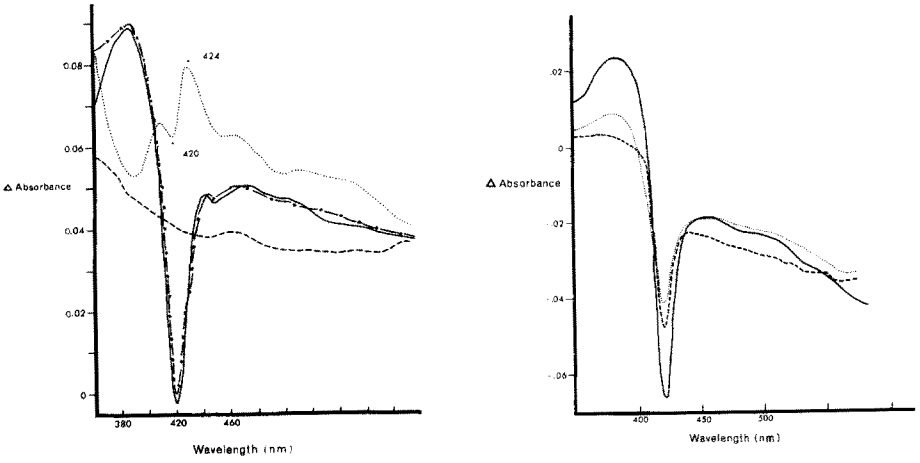

Fig. 2. Displacement of 17-hydroxyprogesterone spectrum with metyrapone. 3 ml of a microsomal suspension (4.0 mg protein/ml) prepared according to the method of SHARMA AND DORFMAN[17] was placed in each of two cuvettes. After recording the baseline (– – – –), several small crystals of 17-hydroxyprogesterone were added (with shaking) to the sample cuvette and the resulting (—) spectral trough recorded. Upon addition of 100 nmoles of metyrapone to the sample cuvette, the spectrum indicated by ····· was evidenced. When metyrap one was added to the reference cuvette a difference spectrum indicated by ●—● was obtained.

Fig. 3. Additive nature of 17-hydroxyprogest erone-induced spectrum to deoxycorticosterone-induced spectrum. Small quantities of deoxycorticosterone crystals were added with shaking to the sample cuvette containing 3 ml of microsomal suspension (4 mg protein/ml) to induce maximal spectral response (confirmed by second addition of small quantities of crystals) with this steroid and the spectrum indicated by – – – – – – recorded. Subsequent addition of small quantities of 17-hydroxyprogesterone crystals extended maximally the spectrum indicated by ———. When the reference cuvette was saturated with deoxycorticosterone by addition of crystals with shaking, the spectrum indicated by ····· was obtained.

progesterone trough was revealed in the same magnitude as the originally recorded trough.

When 17-hydroxyprogesterone is added to a mitochondrial suspension, containing deoxycorticosterone and metyrapone, a significantly lower order of response (less than 5 % on the basis of the spectral peak area) occurs in the diminution of the 425-nm peak in comparison to that observed in the microsomes. This small change likely corresponds with the low order of magnitude at which 17-hydroxyprogesterone is 21-hydroxylated in such mitochondrial preparations.

Characteristics of steroid-induced spectra in carefully prepared microsomes are seen in Fig. 3. Addition of deoxycorticosterone to the sample cuvette results in the induced spectrum indicated by the broken line. Addition of 17-hydroxyprogesterone to a second sample cuvette results in the solid line spectrum. Formation of an identical spectrum as the latter is attained by addition of 17-hydroxyprogesterone to the first sample cuvette, suggesting that deoxycorticosterone does not bind with all receptors capable of associating with 17-hydroxyprogesterone. When the reference cuvette is saturated with deoxycorticosterone, a spectrum indicated by the dotted line tracing occurs which represents the contribution of 17-hydroxyprogesterone over that of deoxycorticosterone when these two steroids are both present in the sample cuvette.

Extension of this observation is shown in Fig. 4. Small increments of 17-hydroxyprogesterone and deoxycorticosterone successively added to sample cuvettes give the values indicated. When the absorbance reading of the sample cuvette containing deoxycorticosterone attained maximum response, 17-hydroxyprogesterone was secondarily added resulting in an extended spectral response as indicated in the

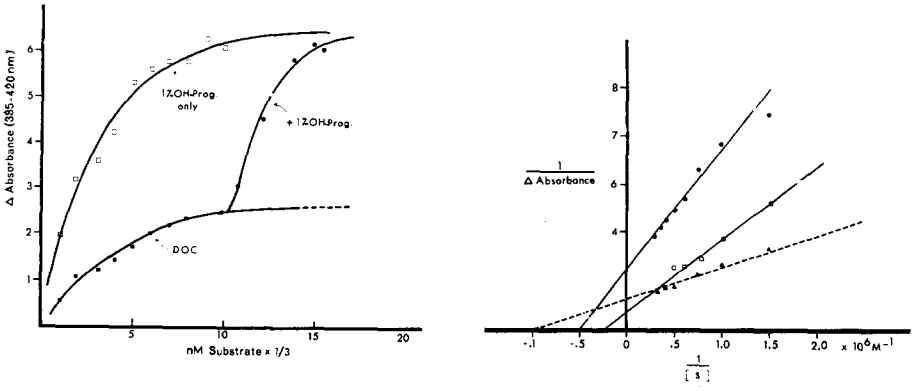

Fig. 4. Quantitated spectral response of deoxycorticosterone and 17-hydroxyprogesterone in adrenal microsomal preparations. A, 17-hydroxyprogesterone was added progressively in increments of 1 nmole (in 0.010 ml ethanol), as indicated on the abscissa of the figure, to the sample cuvette containing 3 ml of microsomal suspension (4.0 mg protein/ml). Control quantities of ethanol were added simultaneously to the reference cuvette and the spectral reading at the time of each addition recorded. These values are indicated by □. B, the same experiment was repeated with comparable quantities of deoxycorticosterone (DOC) in a second sample cuvette (■). C, 17-hydroxyprogesterone was progressively added to B to give the spectral values indicated by ●. For Expt. C, the abscissa values represent total quantities of steroids in the sample cuvette deoxycorticosterone (10 nmoles) *plus* quantity of added 17-hydroxyprogesterone. ----- represents the plateau of the deoxycorticosterone curve obtained in a replica of Expt. B to which solid deoxycorticosterone was added.

Fig. 5. Titration of 17-hydroxyprogesterone (▲), deoxycorticosterone (●) and 17-hydroxyprogesterone in the presence of deoxycorticosterone (□) in adrenal gland microsomal suspensions. The data of this figure were derived from those of Fig. 4.

curve in the upper right of the figure. These data clearly demonstrate the presence of two species of spectral responding receptor groups in microsomes, one complexing specifically with 17-hydroxyprogesterone and one accepting either deoxycorticosterone or 17-hydroxyprogesterone. The complexing constants of these two substrates, derived according to the LINEWEAVER AND BURK[19] methods, are indicated in Fig. 5. A problem was encountered relative to solubility of higher concentrations of 17-hydroxyprogesterone on one hand, and a tendency toward protein precipitation by larger concentrations of deoxycorticosterone on the other. The present investigators have found no indication of a dissociation of the enzyme–steroid complex at high substrate concentrations as reported by WHYSNER AND HARDING[20].

The degree to which the 17-hydroxyprogesterone spectrum cancels the metyrapone spectrum is shown in the experiments of Fig. 6. The initial recording represented by the solid line is that of metyrapone. When 17-hydroxyprogesterone is added simultaneously to both cuvettes (broken line), the 17-hydroxyprogesterone of the reference cuvette generally closely cancels that of the sample cuvette. However, as noted in the figure, an unexplained degree of diminution of the original metyrapone spectrum is at times observed. Capacity of microsomal preparations to respond to these two steroid substrates diminish rapidly after the first day of preparation. Activity is lost both in frozen preparations and preparations stored unfrozen in an ice bath. While there is a progressive loss in capacity for inducing the deoxycorticosterone and 17-hydroxyprogesterone spectra in microsomes, a spectral shift accompanied with a marked increase in magnitude of the 17-hydroxyprogesterone-induced spectrum has been observed in aging of mitochondria. This is likely due to transformation of the mitochondrial cytochrome P-450 to cytochrome P-420. A shift from the 385- to 407-nm peak noted in the mitochondrial spectra is particularly suggestive of such a denaturization. Such a shift and increase in spectral response is not noted in the preparations of the present study with microsomes.

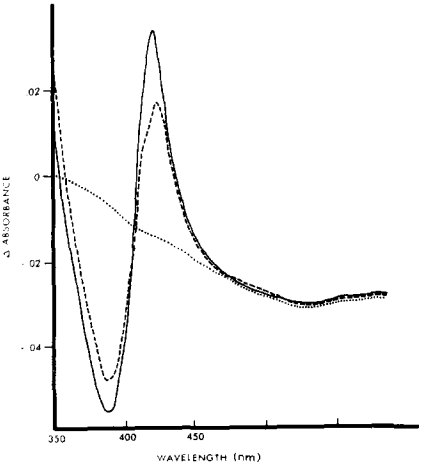

Fig. 6. Spectral displacement of metyrapone spectrum with 17-hydroxyprogesterone. Two 3-ml aliquots of a microsomal suspension (4.0 mg protein/ml) in 0.001 M sodium potassium phosphate buffer were placed in sample and reference cuvettes. Metyrapone (200 nmoles to assure maximum response) was added to the sample cuvette and recorded as ———. When maximum response was assured, 10 nmoles of 17-hydroxyprogesterone were simultaneously added to both the sample and reference cuvette resulting in the spectrum recorded as – – – – –.

Induction of the 17-hydroxyprogesterone trough in the presence of metyrapone has been examined by the LINEWEAVER AND BURK[19] method for determining K_s values as applied by WHYSNER AND HARDING[20] and COOPER *et al.*[21]. The K_s obtained in this manner (Fig. 7) is $5 \cdot 10^{-7}$ M in contrast to that obtained for the induction of the trough in the absence of metyrapone which has been found to be $1 \cdot 10^{-6}$ M in the absence of deoxycorticosterone and $3 \cdot 10^{-6}$ M in its presence. The value, $1 \cdot 10^{-6}$ M, corresponds closely to that of COOPER *et al.*[21] who reported a K_s of $0.7 \cdot 10^{-6}$–$3.8 \cdot 10^{-6}$ M. Values for metyrapone-induced spectra in mitochondria and microsomes are

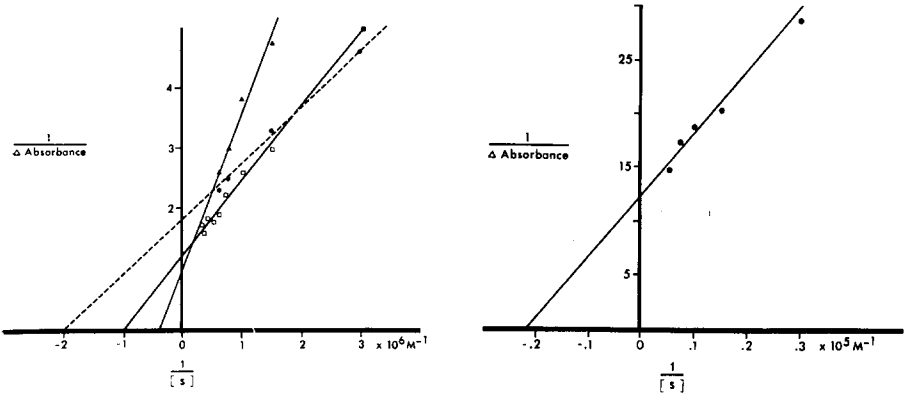

Fig. 7. Characteristics of the induction of the 17-hydroxyprogesterone 420-nm spectral trough in adrenal gland microsomes and adrenal microsomes treated either with deoxycorticosterone or metyrapone. 10 nmoles of 17-hydroxyprogesterone were added in 1-nmole increments of 3 ml of microsomal suspension (4.5 mg protein/ml) and the spectral response examined (after each addition) against an untreated reference microsomal suspension to give the values indicated by □. The same procedure was repeated with 3 ml of microsomal suspension containing 100 nmoles of metyrapone (●). A third sample to which a previous addition of 10 nmoles of deoxycorticosterone had been made was examined (with the addition of 17-hydroxyprogesterone) in the same manner to give the values indicated by ▲.

Fig. 8. Titration of metyrapone in mitochondria. 0.01-ml quantities of 0.01 M metyrapone were successively added to a total of 0.06 ml during which the results of each addition were monitored with the spectrophotometer to give the values indicated. 3 ml of a suspension of washed mitochondria prepared according to SHARMA AND DORFMAN[17], protein equivalent 4.1 mg/ml, was employed.

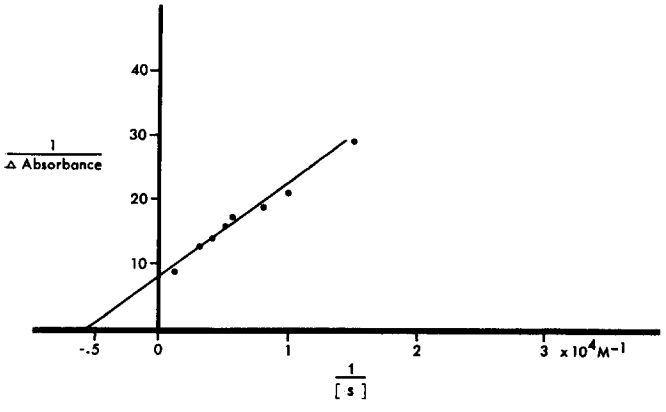

Fig. 9. Titration of metyrapone in adrenal microsomes. The same procedure was employed with a suspension of microsomes (4.5 mg protein/ml) as that for mitochondria in Fig. 8 except the titration was extended to the addition of 0.18 ml of 0.01 M metyrapone.

plotted in Figs. 8 and 9 where the K_s of the metyrapone reaction in mitochondria is found to be $4.6 \cdot 10^{-5}$ and $1.9 \cdot 10^{-6}$ M in microsomes. It is interesting (as yet unexplained) that these values differ between the two cell fractions. They probably relate to the differing nature of the receptor sites.

From the observed substrate-induced spectra and the lack of hydroxylation[1] occurring in these preparations, it must be concluded that the spectra described in this paper relate only to an initial phase of the overall hydroxylation reaction. The overall reaction would seem to be represented

$$S + E \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} ES \underset{k_{-2}}{\overset{k_2}{\leftrightarrows}} EP \underset{k_{-3}}{\overset{k_3}{\leftrightarrows}} E + P$$

(S=substrate; E=enzyme; P=product). Constants observed in this study relate only to the k_1—k_{-1} reaction and should not be associated with values obtained from incubation experiments as reported by other investigators.

The above experiments furnish evidence that the 17-hydroxyprogesterone spectrum, but not the deoxycorticosterone spectrum, is independent of the metyrapone spectrum and that the major change in the observed spectrum upon addition of 17-hydroxyprogesterone is not due to displacement of metyrapone.

DISCUSSION

If the spectral area of the deoxycorticosterone-induced trough of Fig. 3 is represented as "A" and the supplemental area induced by addition of 17-hydroxyprogesterone is represented by "B", it is apparent that 17-hydroxyprogesterone alone can induce a spectral area equivalent to A + B. It must thus be concluded that these two steroids have common (area A) as well as uncommon (area B) characteristics in inducing spectral responses with the pigment receptor mechanisms of microsomal preparations. Whether 17-hydroxyprogesterone displaces deoxycorticosterone from the spectral receptors in these experiments is not known. Reason for the failure of deoxycorticosterone to influence (through summation) the metyrapone-induced spectrum as does 17-hydroxyprogesterone is not clear, especially, as will be discussed below, the complete 17-hydroxyprogesterone spectrum (A + B) does not seem to be implicated with metyrapone. As the deoxycorticosterone response can be a substituent part of the 420-nm spectral trough along with 17-hydroxyprogesterone, it would be expected to influence the reaction similarly in the diminution of the metyrapone spectrum. Partial explanation may reside in a more complex binding mechanism involved for deoxycorticosterone, which not only shares a binding site in common with 17-hydroxyprogesterone but also possibly competes for a second binding site common with metyrapone. A greater affinity of the latter would tend toward making subsequently added deoxycorticosterone unavailable for the A-related receptors. As noted, the degree of diminution of the metyrapone peak with 17-hydroxyprogesterone conforms closely to the area of the 17-hydroxyprogesterone spectrum (A + B) indicating that neither deoxycorticosterone nor metyrapone successfully compete with the 17-hydroxyprogesterone reaction responsible for spectral induction in either the A area or the B area. These data further suggest that the metyrapone-induced spectrum may not be associated directly with the same chemical groupings responsible for the deoxycorticosterone spectral trough. The inverse nature of the two spectra would seem consistent with this concept. An alternate explanation of the above lies in the

possibility that the binding strengths (or a marked difference in the equilibrium state) of the involved receptors (trough) is greatest for 17-hydroxyprogesterone, intermediate for metyrapone (which is not additive with deoxycorticosterone but seems to replace it concurrently with the induction of its own characteristic peak*) and least for deoxycorticosterone. A distinction between these two spectral reactions is further apparent in the observation that deoxycorticosterone induces a spectrum in mitochondria only when cytochrome P-450 is in the oxidized state. Metyrapone gives rise to spectra with either oxidized or reduced cytochrome P-450 (ref. 4). As the 11β-hydroxylating mechanism is implicated in these studies and the specific inhibitor, metyrapone, has little influence on 21-hydroxylation, it would seem that the metyrapone spectral responding receptors may be more closely (and specifically) related to molecular groupings participating in the oxygenation at the C-11-carbon than those manifesting the trough spectrum.

Upon addition of 17-hydroxyprogesterone to a sample cuvette containing metyrapone and comparison against the reference cuvette containing 17-hydroxyprogesterone (Fig. 6), not only is the contribution of the 17-hydroxyprogesterone of the reference cuvette cancelled, but the diminution is observed to extend into the spectral area originally responding to metyrapone. This suggests partial displacement of metyrapone and may represent an equilibrium relationship between the two substrates and their bound complexes. The essentially equivalent area of the 17-hydroxyprogesterone in the sample cuvette cancelled by addition of this substrate to the reference cuvette, along with the spectral shift from 420 toward 425 nm argues for independent enzyme–substrate associations of metyrapone and 17-hydroxyprogesterone.

The inference of NARASIMHULU *et al.*[1] that the 17-hydroxyprogesterone cytochrome P-450 associated induced spectrum at 420 nm relates specifically with microsomal preparations and the inference of COOPER *et al.*[22] that the deoxycorticosterone spectrum relates specifically to mitochondrial preparations is not consistent with either the present or other studies[15] from this laboratory. These investigations reveal that spectral induction by both deoxycorticosterone and 17-hydroxyprogesterone occur in both organelle preparations. The experiments thus invalidate the conclusions of the above referred to laboratories[1,22] based on their data, but support their conclusions on the basis of other parameters. From the present studies it seems conclusive that two distinct species of steroid receptor pigments are present in adrenal organelles. One is predominant in the mitochondria, is significantly present in microsomes and complexes with deoxycorticosterone to form a spectral trough which is deleted by metyrapone. The other occurs predominantly in the microsomes and responds with 17-hydroxyprogesterone to form a spectral trough which exists independently of metyrapone. The supportive spectral effect of the hydroxylatable substrate on the P-450–CO complex as observed by CAMMER AND ESTABROOK[3] closely associates the CO and substrate spectral responses with the same participating pigments.

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* The amplitude of the metyrapone spectrum is the same in either the presence or absence of deoxycorticosterone.

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