Studies on Adrenal Cortical Cytochrome P-450. III. Effects of Carbon Monoxide and Light on Steroid 11β Hydroxylation*

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ABSTRACT: The sensitivity of steroid 11β hydroxylation to carbon monoxide has been examined and compared with the $CO:O_2$ ratio required to produce half-maximal spectrally detectable P-450·CO. The partition value for hydroxylation is about 5 while that for obtaining P-450·CO is about 17. The inhibitory effect of CO on 11β hydroxylation can be released maximally by light at 450 mμ and the action spectrum in this wavelength region indicates that cytochrome P-450 is the oxygen (and CO) binding component functional in this reaction. Assuming a quantum yield of one, studies using intermittent illumination have permitted the calculation of the absolute absorption coefficient of this pigment (β_{450} = 2.5 × 10^6 cm^2/mole of heme) and the generation of its absolute absorption spectrum between 400 and 490 mμ. This spectrum parallels the difference spectrum of P-450·CO except for the presence of a significant peak at 420 mμ in the action spectrum. The basis for the 420-mμ peak in the action spectrum has not been established.

The binding of carbon monoxide to metal-containing compounds resulting in inhibition of the function of the metal and the reversal of this by light at appropriate wavelengths have been extensively studied by Warburg (1949). Estabrook *et al.* (1963) and Cooper *et al.* (1965) using the technique of light reversal of CO inhibition found that a substance combining with carbon monoxide and producing an absorption peak at 450 mμ is involved in the 21 hydroxylation of steroid by adrenal microsomes and the hydroxylation and oxidative dealkylation of several drugs by liver microsomes. They demonstrated that monochromatic light is able to reverse the CO inhibition of these reactions (in the presence of oxygen) and that the effectiveness of this reversal is determined by the wavelength of light used. That is, light at 450 mμ is most effective in releasing the inhibition and the resulting relative photochemical action spectrum for light reversal of the CO-inhibited reaction is approximately the same as the absorption spectrum of the CO-inhibited reaction mixture. As established by Warburg (1949), these results identified cytochrome P-450 as the oxygen binding substance participating in these mixed-function oxidative reactions.

Essentially the same technique was then used by Greengard *et al.* (1967) to demonstrate the involvement of a similar CO-combining substance, absorbing light maximally at 450 mμ, in the 18 hydroxylation of deoxycorticosterone (DOC)[1] in bullfrog adrenal tissue. This report is of interest since steroid 18 hydroxylation occurs within adrenocortical mitochondria as do the complex series of oxidative steps resulting in cleavage of the side chain of cholesterol (producing pregnenolone) and steroid 11β hydroxylation. Previous studies had demonstrated the presence of cytochrome P-450 (P-450) in adrenocortical mitochondria (Harding *et al.*, 1964) and subsequent work has lead to the isolation of two additional components required in 11β hydroxylation. These substances are a NADPH-specific flavoprotein and a nonheme–iron protein (adrenodoxin); they apparently function in a pathway transferring electrons to cytochrome P-450 from NADPH (Omura *et al.*, 1965; Nakamura *et al.*, 1966; Suzuki and Kimura, 1965).

Thus, although it seems likely that P-450 is involved in steroid 11β hydroxylation, specific evidence supporting this view is not available, since the P-450 preparation required in the reconstituted systems of (Omura *et al.*, 1965) is a crude preparation of mitochondrial fragments. A brief report that light at 450 mμ is most effective in reversing CO inhibition of 11β hydroxylation in adrenocortical mitochondria has appeared (Cooper and Rosenthal, 1966) but details and specific data are not available.

The present report describes studies designed to determine the effect of carbon monoxide on the spectrally detectable P-450 and on steroid 11β hydroxylation in adrenocortical mitochondria and to compare these effects. In order to assess the possible involvement of P-450 in this reaction, light reversal of CO-inhibited 11β hydroxylation has also been examined. The resulting absolute absorption spectrum of the CO-binding substance has been determined.

Materials and Methods

Bovine adrenocortical mitochondria were prepared and assays of steroid 11β hydroxylation were performed as described previously (Oldham *et al.*, 1968). Analyzed mixtures of oxygen and nitrogen were obtained from Matheson and known proportions of carbon monoxide and oxygen in nitrogen were obtained from the Scott Research Laboratories. The gas flow was humidified and then fed into erlenmeyer

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[1] Trivial names used are: 11-deoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione (DOC); pregnenolone, 3β-hydroxypregn-5-en-20-one; 11-deoxycortisol, 17,21-dihydroxypregn-4-ene-3,20-dione; cortisol, 11,17,21-trihydroxypregn-4-ene-3,20-dione.

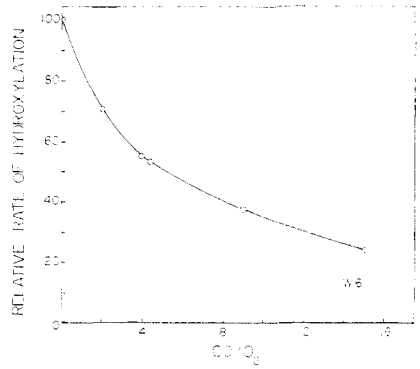

FIGURE 1: Effect of changing ratios of $CO:O_2$ on 11β hydroxylation Mitochondria equivalent to 2.5 mg of N were suspended in phosphate buffer, with cyanide (5 mM) and malate (3 mM) in a final volume of 6 ml. The flask was equilibrated with appropriate $CO–O_2$ mixtures for at least 10 min and the reaction was started by adding 11-[4-^{14}C]-deoxycortisol (250 μM). The rate of 11β hydroxylation in the absence of carbon monoxide was 50 mμmoles/min per mg of N as determined from aliquots removed at 3, 6, and 9 min.

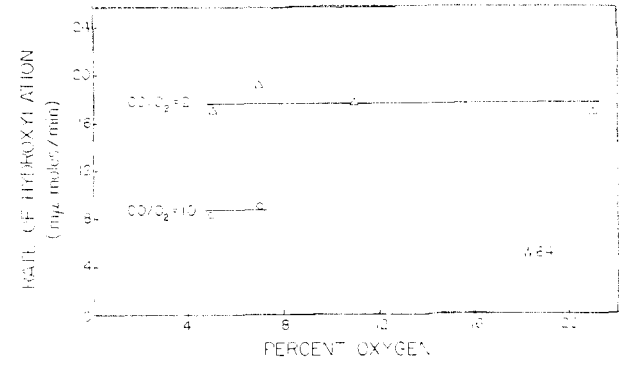

FIGURE 2: Lack of effect of changing O_2 tension on 11β hydroxylation when $CO:O_2$ ratios are constant. Studies were done as described in Figure 1 using mitochondria equivalent to 2.5 mg of N in a final volume of 6 ml. As the oxygen tension in the equilibrating mixture was changed, the CO content was changed appropriately to give constant $CO:O_2$ ratios. Two ratios were used as indicated.

flasks through a glass port in a rubber stopper. The erlenmeyer flasks were painted black except on the bottoms to include most of the incidental light in the room. The gas leaving the first flask was then fed to the second through a similar stopper and port apparatus and ultimately exited into the fume hood system. In all cases two flasks were done simultaneously (in tandem) and in the light studies the second flask served as the dark control since it was not illuminated. The flow of gas into each flask was directed by bent glass tubing and was of sufficient velocity to continually swirl the contents.

Because of the known low light sensitivity of microsomal P-450 (Cooper *et al.*, 1965), a 1600-W xenon lamp was placed in a commercial movie projector aligned on an aluminum optical bench. Approximately 57% of the total light emitted by the lamp was collected by primary and secondary mirrors and the beam was directed to the output lens by wedge shaped lenses, field lenses, and a cylindrical lens. This arrangement results in a nearly collimated beam of quite uniform brightness. Using the arrangement described by Rosenthal and Cooper (1967) the beam leaving the projector housing is passed through a 2% $CuSO_4$ solution, a 2-in. aperture, through narrow band interference filters, and a second $CuSO_4$ solution before entering the blackened aquarium through a 1-in. aperture. The incident beam is then deflected vertically by a 45° first surface mirror onto the bottom of the experimental erlenmeyer flask. The aquarium tank was filled with distilled water in order to maintain constant temperature of about 23°. For any given run temperature was constant to within 1° so that no provision for controlling the temperature was found necessary. Before each light study, the output of the lamp through all the filters and through the glass surfaces was monitored at each wavelength using a wavelength-insensitive bolometer obtained from Yellow Springs Instruments. The bolometer readings were repeatedly checked and calibrated at wavelengths below 400 mμ using the ferric oxalate chemical actinometer described by Calvert and Pitts (1966) and by Seliger and McElroy (1965). The bolometer readings were found to be very reproducible and to be wavelength insensitive between 400 and 440 mμ by this system. For longer wavelengths chemical actinometry was done in some instances using the photoaquation of potassium reineckate as described by Wegner and Adamson (1966). This system was not found to be as convenient as the oxalate system because of the difficulty in preparing the potassium salt without appreciable photodecomposition. However, it gave results in the 400–440-mμ range entirely similar to those obtained with oxalate and provided a check on the bolometric readings at longer wavelengths. Interference filters were purchased from Thin Film Products and were all of the multiple layer type with shorter and longer wavelength blockers cemented on. The half-band width and peak transmission of each filter were determined on a Cary Model 14 spectrophotometer and are given in Table I.

TABLE I: Transmission Characteristics of Interference Filters.

Nominal Peak Position (Å)	True Peak Position (Å)[a]	% Transmission at Peak[a]	Half-Band Width (A)[b]	Relative Window Area[c]
4000	4007	36	62	104
4100	4100	40	34	68
4200	4207	41	39	77
4250	4260	48	41	92
4300	4300	41	33	66
4400	4400	47	43	99
4450	4453	40	21	42
4500	4500	43	46	100
4600	4603	52	47	120
4700	4704	50	47	118
4900	4904	55	52	140

[a] Determined by scanning slowly (against air) in a Cary Model 14 spectrophotometer. [b] Width of the window at the position showing half-maximal transmission. [c] Determined by weighing the area under the transmission curve and comparing with the 450-mμ filter.

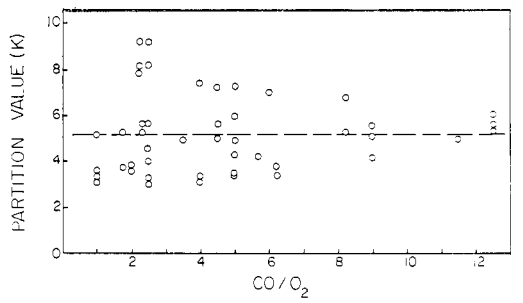

FIGURE 3: Relative constancy of the partition value (K) for 11β hydroxylation as the CO:O_2 ratio is changed. Results are the averages of four experiments in which various, but not all, ratios were used. The partition value is calculated from $K = [n/(1 - n)]CO/O_2$, where n is the rate in the presence of CO divided by the rate obtained in the absence of CO. Oxygen tension varied from 4 to 20%. The broken line represents the average partition value calculated from these data (K = 5.2). The average uninhibited rate of 11β hydroxylation was 72 mμmoles/min per mg of N.

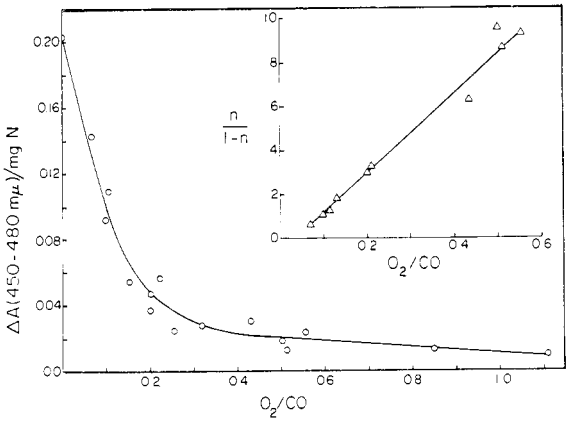

FIGURE 4: Effect of various CO:O_2 ratios on P 450·CO. Values given are averages of four experiments. Mitochondria were suspended in 6.0 ml of phosphate buffer containing malate (5 mM), antimycin A (20 μg) and, sometimes, cyanide (3 mM). The suspension was divided into two cuvets and a base line of equal absorbancy was obtained. The reference cuvet was equilibrated with air while the sample cuvet was emptied into a special 3-port equilibration chamber. This chamber was flushed continuously (while shaking) with the desired gas mixture for at least 10 min and the difference spectrum was run as rapidly as possible (within 2 min). Gas equilibration was repeated if any greater delay occurred. Inset shows a linear curve with slope of K when the data on the ordinate are expressed as the fractional change in absorbancy and plotted against the O_2:CO ratio used.

Because of the variation in the lamp's output at various wavelengths and because of the different transmission character of each of the interference filters, it was necessary to control the intensity of the emitted beam so that the incident light would be quite similar at all wavelengths. Variation in the intensity of the beam was accomplished by using appropriate neutral density filters and more importantly by varying the operating current of the lamp. The lamp's output was found to be almost linear with operating current.

In all studies employing gas mixtures, the incubation flasks were prepared and equilibrated with the gas mixture for at least 10 min prior to the initiation of the reaction of the addition of a very small volume of the steroid substrate. Later experiments used intermittent light, and conditions suitable for obtaining the dark dissociation constant of the carbon monoxide bound P-450 were determined empirically. These are given in the legend to the appropriate figure. Data have been calculated according to Warburg (1949).

Results

In an attempt to determine and compare the effects of CO on 11β hydroxylation and the absorbance at 450 mμ (P-450·CO), numerous studies have been done employing varying proportions of carbon monoxide and oxygen. The results of one such study are shown in Figure 1. Here the relative rate of hydroxylation (compared with the rate obtained in the absence of CO) is plotted against the ratio of carbon monoxide to oxygen used. In all cases the oxygen content was maintained constant at 5%. It is apparent from the curve that 50% inhibition occurred with a ratio CO:O_2 of about 5.

Figure 2 gives data confirming that the degree of inhibition is determined by the ratio of CO to O_2 not just the concentration of carbon monoxide. In this study oxygen tension was varied from 5 to 20% and the carbon monoxide content was altered simultaneously so that a constant CO:O_2 ratio of 2 or of 10 was obtained. Within the error of the method the rate at each ratio is constant.

Data from several such experiments have been grouped together and manipulated arithmetically to obtain the partition constant according to Warburg (1949). This dimensionless constant is equal to the CO:O_2 ratio resulting in a 50% inhibition of the rate obtained in the absence of CO. It can be calculated from the equation: $K = n/1 - n \times CO/O_2$, where n is the ratio of the rate observed in the presence of some proportion of carbon monoxide to that obtained in the absence of carbon monoxide. In Figure 3, K values obtained from four experiments are plotted against the CO:O_2 ratios used. The oxygen content varied from 4 to 20% and it is apparent from the figure that the K value is relatively constant over the entire range of CO:O_2 ratios employed. The values obtained range from about 3 to 9 but despite this variability there was no apparent correlation with the particular CO:O_2 ratio used. The mean K value of about 5.2 is considerably different from partition values of about 1 reported for steroid 21 hydroxylation and for the hydroxylation and N dealkylation of numerous drugs by liver microsomes (Cooper *et al.*, 1965) and from the partition values of 1 to 2.5 reported for steroid 11β hydroxylation by Cammer *et al.* (1968).

Similar and sometimes concomitant studies of the effects of these various gas ratios on the spectrally detectable P-450 (*i.e.*, P-450·CO) have also been done and are summarized in Figure 4. Here the difference in absorbancy between 450 and 480 mμ is plotted against the O_2:CO ratio. A nonlinear relationship is seen and when the data are converted into $n/1 - n$ terms and plotted against the same gas ratio scale (inset), a linear curve is obtained whose scope is K. The average K for the determinations is about 17. In numerous studies K was found to be relatively constant with any given tissue preparation but quite variable with different mitochondrial suspensions. The range of K values was approximately 9 to 20.

CO inhibition of steroid 11β hydroxylation by these mitochondrial preparations can be reversed by light and the

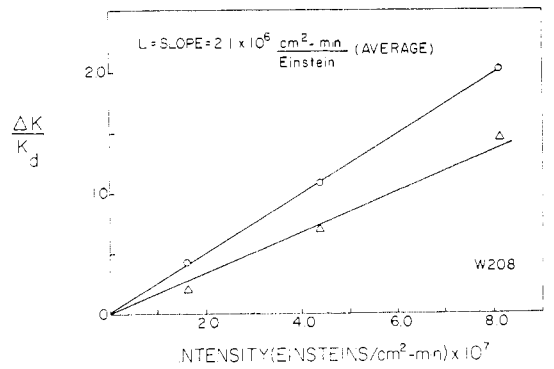

FIGURE 5: Effect of light intensity of CO-inhibited 11β hydroxylation. Studies were done as described in Methods, using light at 450 mμ only and varying the intensity either with neutral density filters and/or changing the operating current of the lamp. $\Delta K = K_L - K_d$ (subscripts refer to light and dark), where $K = [n/(1 - n)]CO/O_2$ and n is the rate obtained with CO divided by the rate observed without CO. The two lines were obtained from different experiments, both employing incubations containing mitochondria equivalent to 1 mg of N, cyanide (4 mM), antimycin A (8 μg/ml), and 11-deoxycortisol in a final concentration of 120 μM. Rates were established from aliquots taken at 1, 2, and 4 min (without CO) or 3, 6, and 9 min (with CO). A nonilluminated control incubation was done simultaneously with each flask subjected to the indicated intensities of illumination. The uninhibited rate was 40 mμmoles/min for the upper curve (open circles) and 46 mμmoles/min for the lower curve (open triangles).

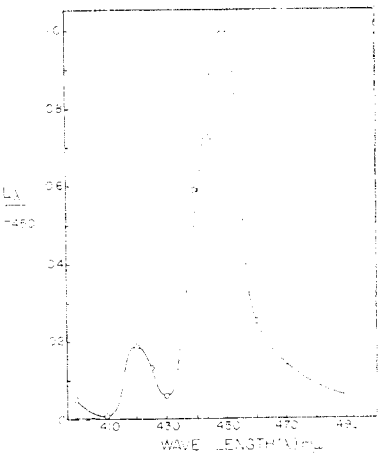

FIGURE 6: Action spectrum for light reversal of CO-inhibited 11β hydroxylation. Mitochondria equivalent to 0.9 mg of N were suspended in 2.5 ml of phosphate buffer containing malate (20 mM), cyanide (4 mM), antimycin A (8 μg /ml), and $NADP^+$ (0.7 mM). Flasks were equilibrated for at least 10 min with a $CO:O_2$ of 8.2. Reaction was started with 11-[4-^{14}C]deoxycortisol (120 μM) and gassing was continued during illumination. A companion dark control was done simultaneously. Rates of hydroxylation were determined from aliquots taken at 3, 6, and 9 min. The ordinate was calculated from $L = [(K_L - K_d)/K_d]1/i$, where the K values are those obtained in light and dark, respectively, and i is the light intensity at the respective wavelengths (Einsteins/cm² min). The uninhibited rate was 18 mμmoles/min.

degree of reversal is dependent on the wavelength as well as on the intensity of light. Figure 5 demonstrates that in two different experiments the extent of reversal is proportional to the intensity of light; light at 450 mμ was used in this case. ΔK is the partition value obtained in the light minus the partition value obtained in the dark. The slope of these lines is equal to the light sensitivity, L, of the system as defined by Warburg (1949). In essence, it is the reciprocal of the intensity of light required to double the dark partition constant. In several studies, L was found to be 2–4 $\times$ 10^6 cm² min/mole quanta. Similar values for the P-450-catalyzed oxidations occurring in microsomes have been reported by Estabrook *et al.* (1963) and by Cooper *et al.* (1965). Using light of different wavelengths, as isolated by the interference filters, it is possible to compare the effectiveness of light by each of the wavelengths in reversing the CO inhibition. A plot of such a study is shown in Figure 6 in which the relative isoquantal effectiveness of the various wavelengths of light is plotted against the wavelengths used. It is clear that light at 450 mμ is most effective in reversing CO inhibition of steroid 11β hydroxylation and that light on either side of this point is considerably less effective. It is of interest, however, that a definite secondary peak of photoactivation occurs at 420 mμ. Since the output of the lamp at these shorter wavelengths is entirely sufficient, it does not appear that this peak is an artifact of the optical procedure. It is true, as noted previously (Table I), that the transmission of light by the filter at 445 mμ is sufficiently small that an inadequate intensity at this wavelength may have been used. It should be noted that in these studies the intensity of light at all other wavelengths was made approximately the same by altering the intensity of the lamp's operating current and/or by using neutral density filters.

In an attempt to obtain the absolute absorption spectrum of the CO-inhibited component, numerous studies were done utilizing intermittent illumination of the CO-inhibited incubation mixture. The basis for this technique is described fully by Warburg (1949) and by trial and error, suitable $CO:O_2$ ratios, illumination periods, and dark intervals were found. The data from one study are summarized in Table II. As indicated, the dark dissociation constant (z_d) of the CO-inhibited component can be calculated from the data given and is estimated to be about 1.2 min^{-1}. If one assumes that the photochemical yield (ϕ) of this reaction is unity (on a heme basis), as was found for all model and natural iron compounds studied by Warburg, it is possible to calculate the absolute absorption coefficient from Warburg's formula; $\beta_\lambda = L_\lambda \times z_d/\phi$. In this case β is the absolute molar absorption coefficient (on the heme basis) and is found to be about 2.5 $\times$ 10^6 cm²/mole of heme. The low value of the molar absorption coefficient is directly due to the low value of L, the light sensitivity. Using the coefficient obtained at 450 mμ, the relative absorption spectrum of the CO-combining species can be converted into the absolute absorption spectrum by multiplying the ordinate in Figure 6 by 2.5 $\times$ 10^6. The result is illustrated in Figure 7.

Discussion

The data reported here for the partition value of steroid 11β hydroxylation do not agree with a preliminary report by Cooper and Rosenthal (1966) or the data presented by Cammer *et al.* (1968) for the same reaction. Those investigators found a value of 1 to 2 in agreement with reported values for steroid 21 hydroxylation in adrenal microsomes (Esta-

TABLE II: Intermittent Light on 11β Hydroxylation.

$CO:O_2$	Light Period (τ)	Dark Period (ν)	Rate of Hydroxylation (mμM/min)	Light Intensity at 450 mμ (Einstein/cm^2 min)	n[a]	z_d[b] (min^{-1})
8.2	0	All	10.5	0	0.39 (n_d)	
0	All	0	27.0	0.81×10^{-6}		1.20
8.2	All	0	16.5	0.81×10^{-6}	0.61 (n_L)	
8.2	1.5 min	1.5 min	14.1	0.81×10^{-6} during τ		

[a] Determined from n = rate in presence of CO/rate in absence of CO. n_d is the value obtained in the dark and n_L was obtained during constant illumination. [b] Dark dissociation constant, calculated according to Warburg (1949).

brook *et al.*, 1963) and the hydroxylation of acetanilide and oxidative dealkylation of several drugs by liver microsomes (Cooper *et al.*, 1965). The reason for these reported differences in carbon monoxide sensitivity of 11β hydroxylation is not known. There seems to be no compelling reason why other mixed function oxidases should have the same carbon monoxide sensitivity. Wada *et al.* (1967b, 1969) have presented evidence that cholesterol synthesis by liver microsomes has a partition value of 6 to 50 depending on the precursor used. In this tissue Wada and coworkers (1967a) have also reported that the ω oxidation of stearic and lauric acids has a partition constant of about 0.1. A considerable variation in the CO sensitivity of the spectrally detectable P-450 has also been reported. Omura and Sato (1964) present data on P-450 from liver microsomes which allows calculation of the partition value. This is about 20 whereas Estabrook *et al.* (1963) report a value of 1 to 2 for P-450 in adrenal microsomes. An extensive study of the CO sensitivity of liver microsomal P-450 by Ichikawa *et al.* (1967) indicates that the partition "constant" varies from 15 to 66 with decreases in pH. These investigators also found that there was no significant difference in CO sensitivity between liver and adrenal microsomal P-450.

No attempt was made in our studies to relate the partition value of either 11β hydroxylation or P-450·CO to pH. Both were determined at the same pH and frequently using the same fresh mitochondrial preparation. It is recognized that partition constants may not be constant at all (Warburg, 1949; Rosenthal and Cooper, 1967) and that, if the rate of electron transport is limiting, the partition value of the same preparation will vary depending on the $CO:O_2$ ratio used. Such a variation was found occasionally but even in these cases the K value for 11β hydroxylation was never below 3. Also, a spectral K value of less than 7 was never obtained. The explanation for the difference in CO sensitivity of 11β-hydroxylation and the spectrally measureable P-450 is not known although it is true, of course, that hydroxylation requires turnover of P-450 while formation of P-450·CO does not. This difference in CO sensitivity may then reflect differences in steady-state and equilibrium values. An alternative possibility is that 11β hydroxylation uses only one P-450 species while the spectral determination measures several different species, most having higher partition values than the species involved in 11β hydroxylation.

The light reversal studies demonstrate that P-450 is the oxygen-activating component in 11β hydroxylation, since no other CO-binding substance is present which absorbs light maximally at 450 mμ. The small peak at 420 mμ indicates that light of this wavelength is also effective in reversing the CO inhibition. The effectiveness of light at 420 mμ was quite variable although a definite peak was always found at this wavelength. Such a peak in the action spectrum of mixed-function oxidase has not been previously described but it is not certain that other studies used sufficient light at these lower wavelengths (Estabrook *et al.*, 1963). Simpson and Boyd (1967) describe a 410- and 490-mμ peak (in addition to the principal 450-mμ maximum) in the action spectrum for cholesterol side chain cleavage. Also, indications of reasonably effective light at wavelength less than 450 mμ can be found in the data given by Cooper *et al.* (1965) for codeine demethylation by liver microsomes. While it seems clear that the 450-mμ peak is caused by P-450 (*i.e.*, P-450·CO), the source of the 420-mμ peak in the action spectrum is unknown. The

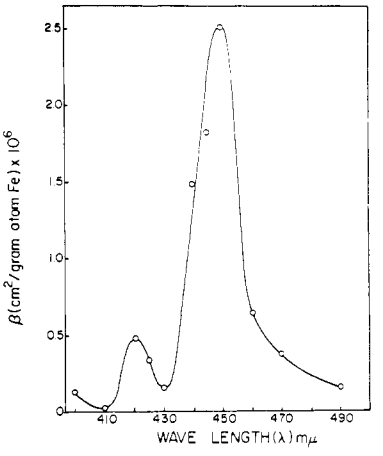

FIGURE 7: Absolute absorption spectrum of CO-inhibited pigment required in 11β hydroxylation. The relative action spectrum (Figure 6) was converted into the absolute spectrum by multiplying the ordinate by the calculated absolute absorption coefficient at 450 mμ ($\beta = 2.5 \times 10^6$ cm^2/mole of heme). β_{450} was calculated from $\beta_{450} = L_{450}/z_d/\phi$, assuming the quantum yield (ϕ) to be 1. Calculation of z_d was done according to Warburg (1949) from data given in Table II.

CO-difference spectrum of these particles shows a peak of 450 mμ and a variable absorption at 420 mμ apparently related to hemoglobin contamination and/or to cytochrome P-420. Although neither is thought to be active in hydroxylation, it is possible that energy absorbed by either of these hemes could be transferred to P-450·CO resulting in activation of hydroxylation. It is also possible that P-420 is capable of participating in 11β hydroxylation or that P-450·CO has a split Soret absorption. While this kind of spectrum would be unusual for *b*-type cytochromes, it is known that liver microsomal P-450 has two Soret bands (430 and 455 mμ) when reduced and combined with ethyl isocyanide and, further, that the relative magnitudes of the two bands are dependent on pH (Nishibayasbi *et al.*, 1966; Imai and Sato, 1967).

The sensitivity of a CO-inhibited component to light is characteristic of that component. The results reported here show that the light sensitivity at 450 mμ of P-450·CO involved in 11β hydroxylation is 2 to 4 $\times$ 10^6 cm^2 min/mole quanta. The linear curves passing through the origin (Figure 5) indicate that the system is reasonably described by the equation and the assumptions made by Warburg in deriving this relationship (1949). This value of L is slightly greater than that reported by Estabrook *et al.* (1963) and Cooper *et al.* (1965) for microsomal P-450-catalyzed oxidations. As noted by those investigators, these values for the light sensitivity are about two orders of magnitude lower than for any heme studied by Warburg. It is in the same range as the light sensitivity of a ferrocysteine (nonheme) model as reported by Warburg (1949).

Determination of the dark dissociation constant of P-450·CO has been done empirically according to Warburg's procedure (1949). The procedure finally established allowed calculation of the various constants described by Warburg and permitted an evaluation of the validity of his assumptions applied to 11β hydroxylation. These were found to be reasonably well satisfied (switching from dark to light is very rapidly accompanied by a shift in the dark partition value to the light value and a light-to-dark switch resulted in a slow and incomplete transition to the dark partition value). The dark dissociation constant obtained (1.2 min^{-1}) is somewhat greater than the values found by Warburg for many of the reactions studied by him. Using this value and the assumption that the photochemical quantum yield is unity at the wavelengths studied, it was possible to calculate the absolute absorption spectrum of the CO-absorbing species. The result obtained (Figure 7) may require revision in the region of 420 mμ if it is subsequently found that this absorption band is not an intrinsic property of P-450·CO as discussed earlier.

It is difficult—if not impossible—to relate the absolute absorption coefficient to published data since quantitative studies have used difference spectrophotometry (Omura and Sato, 1964; Horie *et al.*, 1966; Kinoshita *et al.*, 1966; Mitani and Horie, 1969). The "absolute" spectra reported still involve subtraction of highly scattering contents in a reference cuvet. Recent studies by Mitani and Horie (1969) have provided some data on the absolute absorption of partly purified P-450·CO. From their data an absolute molar absorption coefficient for the Soret maximum of P-450·CO can be estimated to be about 10% the value reported here (and three orders of magnitude less than other hemes). The reasons for this discrepancy are not known since it seems unlikely that the true quantum yield is 10 (it was assumed to be unity) and it is also doubtful that either the light sensitivity or the dark dissociation constants has been estimated to be *high* by an order of magnitude. Verification of the absolute absorption spectrum of the CO-inhibited component in steroid 11β hydroxylation will require isolation and purification of the hydroxylating cytochrome.

Added in Proof

A recent report by Diehl *et al.* (1969) clearly describes the presence of a peak at 420 mμ, in addition to the peak at 450 mμ, in the photochemical action spectrum obtained in light reversal of CO-inhibited cyclohexane hydroxylation by rat liver microsomes.

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Studies on Adrenal Cortical Cytochrome P-450. IV. Effects of Carbon Monoxide and Light on Cholesterol Side Chain Cleavage*

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ABSTRACT: The oxidative cleavage of the side chain of cholesterol has been studied in extracts of acetone powders prepared from bovine adrenocortical mitochondria. Some of the properties of these powders are described and, it is found that side chain cleavage is 50% inhibited with a $CO:O_2$ ratio of about 1 and that light at 450 mμ is best able to reverse this inhibition. However, no evidence of peaks in addition to that at 450 mμ in the action spectrum could be demonstrated. By using intermittent illumination and assuming a quantum yield of unity, the absolute absorption coefficient at 450 mμ has been determined ($\beta_{450} = 3.9 \times 10^6$ cm^2/mole of heme). The absolute absorption spectrum of the CO-inhibited component (P-450) from 400 to 490 mμ has been calculated. Comparison of these results with those found for 11β hydroxylation suggests that a different P-450 is used for each of these reactions.

The side chain cleavage of cholesterol occurs in adrenocortical mitochondria (Halkerston *et al.*, 1961; Constantopoulos and Tchen, 1961a) and is thought to involve at least two successive hydroxylations (Solomon *et al.*, 1956; Shimizu *et al.*, 1961, 1962; Constantopoulos *et al.*, 1962; Constantopoulos and Tchen, 1961b). The first appears to be the 20α hydroxylation of cholesterol followed by 22ϵ hydroxylation. Side chain scission then results, perhaps *via* another oxidative reaction (Constantopoulos *et al.*, 1962). Evidence is available suggesting that the 20α hydroxylation of cholesterol is the rate-limiting step in corticosteroid synthesis and, as the slow step, represents the point of stimulation by ACTH (Stone and Hechter, 1955; Karaboyas and Koritz, 1965). It is apparent that each of the hydroxylation reactions occurring in adrenal mitochondria (11β, 18, 20α, and 22ϵ) is quite specific and yet evidence to date indicates no component which is unique to any of these particular reactions.

Ichii *et al.* (1967a,b), have fragmented adrenocortical mitochondria and have found that a NADPH-specific flavoprotein and adrenodoxin both stimulate cholesterol side chain cleavage in these partly purified preparations. Each of these components was thought to be essential for the activity of both 20α hydroxylation and the subsequent 22ϵ hydroxylation. However, in these studies the rates of the various reactions could not be correlated with the content of cytochrome P-450 (P-450) nor was there parallelism between the amount of P-450, its specific activity or the activity of either hydroxylation reaction during progressive stages of purification. Similar studies have been reported by Bryson and Sweat (1968) who found that cholesterol side chain cleavage is most active in the fully reconstituted system (flavoprotein, adrenodoxin, and crude P-450). This system was found to be severely inhibited by carbon monoxide.

Young and Hall (1968, 1969) have reported that the side chain of cholesterol sulfate can be cleaved by adrenal mitochondria forming pregnenolone[1] sulfate. This process was not inhibited by substrate amounts of cholesterol even though it appeared to require the P-450 reductase system (NADPH, the NADPH-specific flavoprotein, adrenodoxin, and a crude preparation of cyrochrome P-450).

The similarity of cholesterol side chain cleavage to 11β hydroxylation would appear to be considerable in that each occurs in adrenal mitochondria, each requires a NADPH-specific flavoprotein, oxygen, adrenodoxin, and each is inhibited by carbon monoxide. Previous results from this laboratory (Wilson and Harding, 1970) have demonstrated the requirement for a 450-mμ-absorbing species in steroid 11β hydroxylation. The involvement of a similar 450-mμ-absorbing species (cytochrome P-450) in the overall side chain cleavage of cholesterol has been reported by Simpson and Boyd (1967). However, the action spectrum published by these workers shows areas dissimilar to the observed difference spectrum of P-450·CO and, in addition, these

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[1] Trivial names used are: pregnenolone, 3β-hydroxypregn-5-en-20-one; cortisol, 11,17,21-trihydroxypregn-4-en-3,20-dione; 17-hydroxyprogesterone, 17-hydroxypregn-4-ene-3,20-dione; 11-deoxycortisol, 17,21-dihydroxypregn-4-ene-3,20-dione.