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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.

TABLE I: Properties of Acetone Powders.

Tissue	mg of N/ml	μg of Cholesterol/mg of N		P-450, mμmoles/mg of N	11β Hydroxylation, mμmoles/mg of N min	Side Chain Cleavage, mμmoles/mg of N min
		Total	Free			
Mitochondria	8.9	147	45	6.0	69	0.02
Supernatant[a]	3.1	16	4	0.6	0.1	4.7
Sediment[a]	5.1	80	42	4.2	0.01	0.2

[a] Obtained by homogenizing the acetone powder in 0.16 M phosphate buffer (pH 7.2) and centrifuging at 105,000*g* for 90 min. The sediment was resuspended in 0.25 M sucrose.

investigators describe preparations quite active in the side chain cleavage of cholesterol containing no detectable P-450 by conventional difference spectrophotometry.

The present study examines the carbon monoxide sensitivity of cholesterol side chain cleavage and the light sensitivity of the CO inhibition of this reaction. The resulting action spectrum for light reversal of CO inhibition parallels the difference spectrum of carbon monoxy·P-450, establishing the involvement of P-450 in the reaction, and the absolute absorption spectrum of this substance has been determined.

Materials and Methods

Bovine adrenocorticol mitochondria were prepared as previously described (Oldham *et al.*, 1968) and extracts of acetone powders derived from these mitochondria were prepared as described by Halkerston *et al.* (1961) or as described by Kimura *et al.* (1966). Assays of cholesterol side chain cleaving activity were performed in phosphate buffer (pH 7.2) using $^{14}C_4$-labeled cholesterol as substrate. The radioactive cholesterol was mixed with appropriate quantities of unlabeled cholesterol and emulsified by sonication in aqueous 1% (w/v) lecithin. NADPH or a NADPH-generating system (NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase) was used as the source of electrons and gassing was done as previously described (Wilson and Harding, 1970). Aliquots of the incubation mixture were removed at various times, quenched in 10 volumes of ethyl acetate containing unlabeled progesterone and unlabeled cholesterol, and reextracted with 5 volumes of chloroform. The organic extracts were combined, reduced in volume, and then chromatographed on paper impregnated with propylene glycol and developed with cyclohexane saturated with propylene glycol. After drying, progesterone was located by its ultraviolet absorption and the strips were scanned with a commercial radiochromatogram scanner. Quantitation was carried out by electronic integration of the peak areas. With this system cholesterol, pregnenolone, and progesterone had R_F values of about 0.9, 0.3, and 0.5, respectively. Using the acetone powder preparation pregnenolone was the dominant product by far although small quantities of progesterone were also formed. Since cholesterol moves at or near the front in this chromatography system, less polar metabolites of this substance are not adequately separated. However, in numerous examinations using the heptane–phenylcellusolve system of Neher and Wettstein (1952), no metabolites or products less polar than cholesterol were ever found. Products more polar than pregnenolone were rarely obtained using the acetone powder but these products were commonly observed when whole mitochondria were employed. No special attempt to identify these metabolites was made, although in suitable chromatography systems these substances had mobilities similar to 17-hydroxyprogesterone, 11-deoxycortisol, and cortisol. Numerous attempts to identify 20α-hydroxycholesterol and the dihydroxylated cholesterol product were unsuccessful. This failure is in accord with reports by several investigators (Halkerston *et al.*, 1961; Koritz and Hall, 1964; Simpson and Boyd, 1967)

The apparatus and procedures for the gassing and light studies have been previously described (Wilson and Harding, 1970). Measurement of steroid 11β hydroxylation and P-450 content were done as described previously (Oldham *et al.*, 1968). Free and esterified cholesterol were measured according to the procedure of Webster (1962). The method of data analysis and the assumptions made have been discussed by Warburg (1949).

Results

Numerous attempts to perform these studies using intact mitochondria with cholesterol as a substrate were variably successful but quite inconsistent. It was not possible to determine with certainty the absolute rate of cholesterol cleavage in the intact particles because of the large amount of free and esterified cholesterol already present. Subsequently it was found that aqueous extracts of acetone powders of adrenocortical mitochondria contained essentially no cholesterol and yet retained significant cholesterol side chain cleavage activity. In fact, these extracts were manyfold more active in cleaving the side chain from cholesterol than was the intact mitochondrial preparation and the rate of this activity is comparable with the fastest rate reported in the literature (Kimura *et al.*, 1966). A comparison of the properties of the extracted acetone powders with intact mitochondria and with the fraction of the powders sedimented after extraction is given in Table I. Steroid 11β hydroxylation is seen to decrease drastically with treatment by acetone. Similarly the content of P-450 is only about 0.1 as much in the supernatant as it is in the mitochondria. A similar decrease in free and esterified cholesterol is also noted. Although the extracted

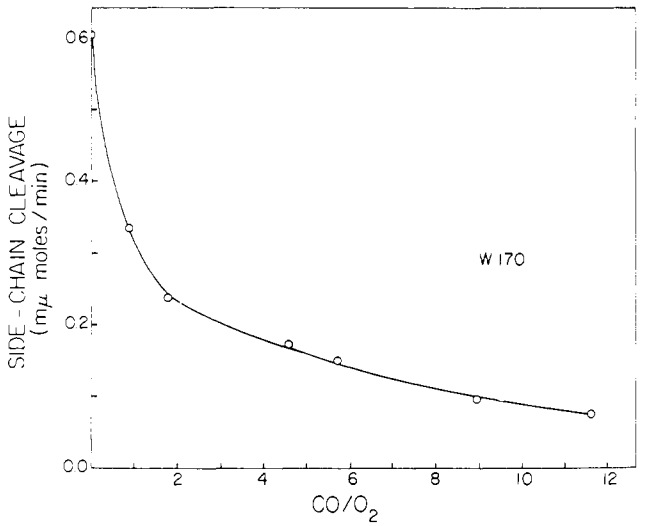

FIGURE 1: Effect of various $CO:O_2$ ratios on cholesterol side chain cleavage. Tissue equivalent to 0.09 mg of N (sediment) and 0.25 mg of N (supernatant) was incubated in phosphate buffer after equilibration with the desired gas mixtures for at least 10 min. Oxygen content varied from 7 to 15%. Aliquots were removed at 3 and 6 min for analysis as described in Methods.

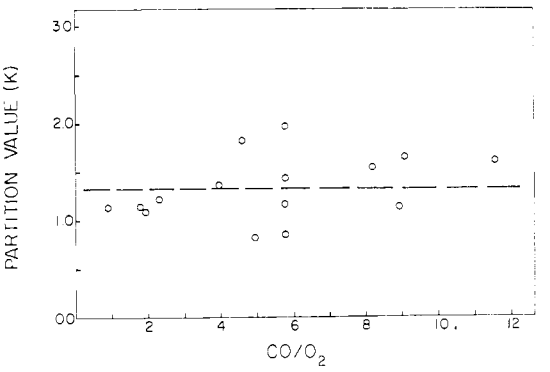

FIGURE 2: Effect of different $CO:O_2$ ratios on the partition value of cholesterol side chain cleavage. The data were obtained from four experiments, all using extracts of acetone powders and performed as described in Figure 1. The average rate of side chain cleavage in the four experiments was 2.4 mμmoles/min per mg of N in the absence of carbon monoxide. The partition values were calculated as described in the text. The broken line indicates the average value for all points ($K = 1.3$).

residue of the powder has considerable P-450, its 11β-hydroxylation activity and side chain cleaving activity are quite reduced. Since the activities of NADPH–dichlorophenolindophenol reductase and NADPH–cytochrome *c* reductase activities are 8–20-fold greater in the supernatant than in the sediment (data not shown), it seems likely, as suggested by Yago and Ichii (1969), that the extracted sediment of such acetone prepared powders is deficient in adrenodoxin. This would account for the low activities of both hydroxylation reactions and the reductases in this fraction despite the presence of considerable P-450.

Additional studies of the cholesterol side chain cleaving system in these acetone powder preparations (and not reported in detail here) reveal an approximate K_m for cholesterol of 5–15 μM and a NADPH optimum of about 20 μM when using glucose 6-phosphate and glucose 6-phosphate dehydrogenase as a generating system.

The effect of carbon monoxide on cholesterol side chain cleavage in these extracts is shown in Figure 1 in which the absolute rate of cleavage is plotted against the $CO:O_2$ ratios used. In these studies, the oxygen content varied from 7 to 15%, and one can calculate partition values (K) for each of the points from the equation $K = [n/(1 - n)]CO/O_2$. In this equation, n is equal to the rate of side chain cleavage in the presence of some CO–O_2 mixture divided by the rate obtained in the absence of carbon monoxide. A number of such studies have been done, the results combined and the partition values calculated. These results are shown in Figure 2 where the variation in K with changing $CO:O_2$ ratios is plotted. Although considerable scatter of the points is seen, it is apparent that the partition value varies only from 0.8 to 1.9 as the gas ratio varies from 1 to 12. The mean partition values for all of these points is 1.3 which contrasts significantly with the previously demonstrated partition value for steroid 11β hydroxylation of about 5 (Wilson and Harding, 1970). It must be admitted that the tissue preparation used for each of the two reactions is different (extracts of acetone powder and intact mitochondria) and therefore a comparison of the partition values may not be valid. However, in a few experiments using extracted acetone powders and assaying for 11β hydroxylation, partition values lower than 5 were never obtained. This suggests that the difference in partition values between 11 hydroxylation and cholesterol side chain cleavage is not explainable on the basis of different tissue preparations. The value for cholesterol side chain cleavage is quite similar to that previously recorded by Simpson and Boyd (1967). It is also similar to values reported for steroid 21 hydroxylation (Estabrook *et al.*, 1963) and for various mixed-function oxidases occurring in liver microsome (Cooper *et al.*, 1965)

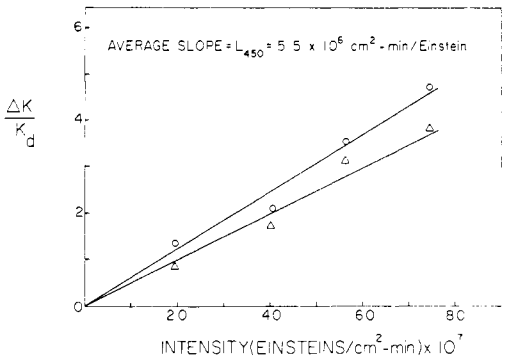

FIGURE 3: Effect of light intensity at 450 mμ on the partition constant. Tissue equivalent to 0.3 mg of N (0.18 mg of N supernatant and 0.12 mg of N sediment) and a $CO:O_2$ ratio of 5.0 were used. Light intensity at 450 mμ was varied by employing neutral density filters or changing the operating current 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 in which rates were determined from aliquots were removed at 4 and 8 min. For the upper curve (open circles) the uninhibited rate was 0.64 mμmole/min and K_d was 1.3. In the experiment shown by the open triangles the uninhibited rate was 0.56 mμmole/min and K_d was 1.1.

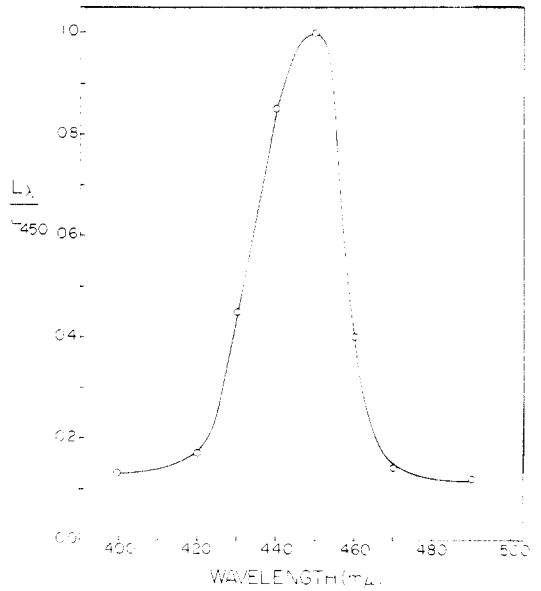

FIGURE 4: Relative action spectrum for light reversal of CO-inhibited side chain cleavage. The plot shows the average of three experiments done as described in methods.

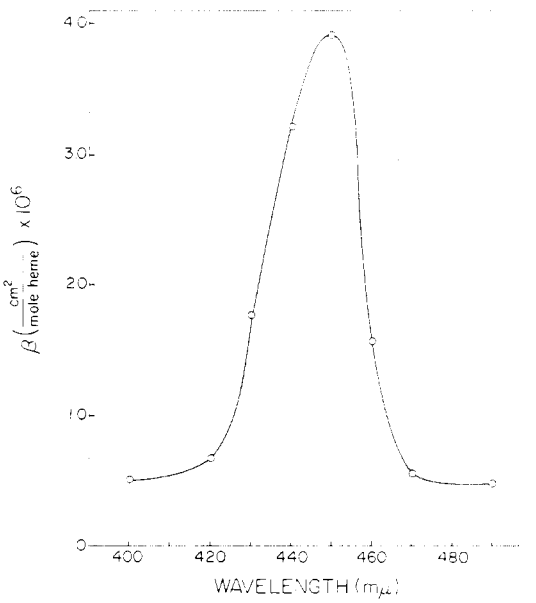

FIGURE 5: Absolute absorption spectrum of CO-inhibition component required in side chain cleavage. Intermittent light studies were done with estimations of the rate of side chain cleavage. The dark dissociation constant (z_d) found was 0.7 min^{-1} allowing calculation of β from $\beta_{450} = L_{450} \times z_d/\phi$. The relative absorption spectrum (action spectrum) given in Figure 4 was then correct using this value of β at 450 mμ.

but is three- to fourfold greater than the partition value calculable from data presented by Young and Hall (1969) for the side chain cleavage of cholesterol sulfate.

As reported by Simpson and Boyd (1967), the CO inhibition of cholesterol side chain cleavage can be reversed by light and Figure 3 shows the effect of the intensity of light at 450 mμ on this reversal. The results indicate that the extent of reversal at this wavelength is proportional to the light intensity over a reasonably wide range and allows a calculation of the light sensitivity at 450 mμ. The light sensitivity (L) is the reciprocal of the light intensity required to double the dark partition value. In Figure 3, L_{450} is the slope of the line and is found to be about 5.5×10^6 cm^2 min/mole quanta. This value is somewhat higher than the values found for steroid 11β hydroxylation (Wilson and Harding, 1970) and steroid 21 hydroxylation (Estabrook *et al.*, 1963).

The photochemical action spectrum obtained for the light reversal of CO-inhibited side chain cleavage is shown in Figure 4. The light sensitivity obtained at each wavelength relative to the light sensitivity at 450 mμ is plotted against wavelength and the curve demonstrates a smooth maximum at 450 mμ. Unlike the action spectrum obtained for steroid 11β hydroxylation (Wilson and Harding, 1970), there are no secondary bands apparent and the spectrum is reasonably symmetrical about the rather broad peak at 450 mμ. This spectrum is quite similar to those obtained in microsomes by Cooper *et al.* (1965) but differs from that reported by Simpson and Boyd (1967) for cholesterol side chain cleavage since the secondary peaks at 410 and 490 mμ obtained by those workers are not confirmed by Figure 4.

Intermittent illumination of the reaction mixture at 450 mμ has been done in a manner similar to that previously described for steroid 11β hydroxylation (Wilson and Harding, 1970). The dark dissociation constant (z_d) was calculated to be about 0.7/min and from several studies a mean light sensitivity value of about 5.5×10^6 cm^2 min/mole quanta was obtained at 450 mμ. Combining these two results with an assumed photochemical quantum yield of unity ($\phi = 1$), the absolute molar absorption coefficient at 450 mμ has been calculated as described by Warburg (1949) from $\beta_{450} = L_{450} \times z_d/\phi$. This value ($\beta_{450}$) is about 3.9×10^6 cm^2/mole of heme. Using this, the relative absorption spectrum (photochemical action spectrum given in Figure 4) can be converted into the absolute absorption spectrum as shown in Figure 5.

Discussion

The use of acetone powders of adrenal mitochondria helped solve some problems but also created new ones. Attempts to relate 11β hydroxylation activity and P-450 content in these powders to cholesterol side chain cleavage were largely unsuccessful. The production of cortisol from 11-deoxycortisol by these extracted powders is very slow while in intact mitochondria this rate is more than 100-fold greater than side chain cleavage. It appears that full activity of 11β hydroxylation in the extracted powder is limited by the loss (or destruction) of a necessary component during preparation. A similar effect on side chain cleavage is also possible but perhaps would not be observed since apparent activity in the intact mitochondria is so low. The enhanced rate of side chain cleavage in the powders is probably related to two factors; the loss of permeability barriers to the added radioactive cholesterol and the removal of large amounts of endogenous cholesterol by acetone extraction.

In any case it is clear, as reported by Simpson and Boyd (1967), that the production of pregnenolone from cholesterol is inhibited by carbon monoxide. The partition value is about one, in agreement with these investigators and with reports

by Estabrook *et al.* (1963) and Cooper *et al.* (1965) for P-450-catalyzed oxidations in microsomes. This result contrasts significantly with the partition value of five found for steroid 11β hydroxylation (Wilson and Harding, 1970). It is also much less than the spectral partition constant found for P-450·CO in adrenal mitochondria (Wilson and Harding, 1970) and in adrenal and liver microsomes (Ichikawa *et al.*, 1967). Although the basis for these differences is not known, the simplest explanation is that several different varieties of P-450 exist.

In agreement with this suggestion is the finding of qualitative differences between the photochemical action spectra of 11β hydroxylation (Wilson and Harding, 1970) and of the cholesterol side chain cleanage. In disagreement with results by Simpson and Boyd (1967), the spectrum reported here for side chain cleavage has no secondary peaks. The curve is relatively symmetrical about the broad 450-mμ peak indicating that P-450 is the oxygen-activating (and carbon monoxide inhibited) component. It is interesting that the CO-difference spectrum of these extracts has a significant absorption band at 420 mμ (caused apparently by hemoglobin and/or P-420) as well as at 450 mμ. Despite this, no photoactivation peak at 420 mμ could be demonstrated, unlike the findings with 11β hydroxylation (Wilson and Harding, 1970).

The light sensitivity of the P-450·CO active in the cleavage of cholesterol's side chain is somewhat higher than values reported for other reactions (Wilson and Harding, 1970; Estabrook *et al.*, 1963; Cooper *et al.*, 1965). The significance of this finding is uncertain since the error in this determination—here and in the literature—has not been estimated. Nevertheless, the light sensitivity is still much less than values found by Warburg (1949) for a number of heme compounds and is similar only to other P-450-catalyzed reactions (and to a ferrocysteine model studied by Warburg). It seems to be characteristic of P-450·CO. Since the estimated dark dissociation constant is about 0.7 (min^{-1}) and since the quantum yield has been assumed to be unity, it is clear that the low molar absorption found at 450 mμ (β_{450}= 3.9×10^6 cm^2/mole of heme) is directly due to this small light sensitivity.

These results indicate that cytochrome P-450 is the oxygen-binding and CO-inhibited component involved in the side chain cleavage of cholesterol. The magnitudes of the absolute absorption coefficients obtained at 450 mμ for this reaction and for 11β hydroxylation (Wilson and Harding, 1970) are similar. Despite this, the significant differences observed in the CO sensitivity and in the action spectra (at 420 mμ) suggest that different species of P-450 are functional in these two reactions.

Since the side chain cleavage of cholesterol appears to be a series of at least three reactions, the specific reaction(s) inhibited by carbon monoxide has not been established. From the data as discussed earlier it is assumed that the limiting step in the overall process is CO sensitive (or inhibition would not be apparent) and that the initial 20α-hydroxylation step is the one affected. The inability to isolate radioactivity labeled 20α-hydroxycholesterol in these studies supports this assumption. However, this (and other data) is indirect and studies dealing with the CO sensitivity and light reversal of the inhibited individual reactions will be required to establish this point.

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