THE EFFECT OF STEROID HYDROXYLASE INHIBITORS ON THE RATE OF REDUCTION OF ADRENAL MITOCHONDRIAL CYTOCHROME P-450*

E. Noel McIntosh and Hilton A. Salhanick

Center for Population Studies and Department of Obstetrics and Gynecology, Harvard University, Boston, Massachusetts 02215

Received June 20, 1969

When added to adrenocortical mitochondria, metyrapone, amphenone and aminoglutethimide induce type II difference spectra, and block both the initial rate of malate-linked cytochrome P-450 reduction and also the DOC-induced increase in this rate. Removing the $-NH_2$ group from aminoglutethimide shifts the spectrum to shorter wavelengths and reduces its effectiveness in blocking P-450 reduction. These compounds appear to block steroid hydroxylation by binding to oxidized P-450 and only very slowly allowing the P-450 to be reduced.

In adrenocortical mitochondria reducing equivalents derived from intramitochondrial NADPH are transferred to cytochrome P-450 and, by a still incompletely understood mechanism, are utilized in steroid hydroxylation reactions. An essential feature of these reactions presumably is the initial formation of a substrate-oxidized cytochrome P-450 complex. Recently, Gigon et al (1,2) working with the hepatic microsomal mixed-function oxidase system have correlated the rate of reduction of P-450 with the type of spectral change resulting from this interaction. Thus compounds inducing a type I[1] difference spectra accelerate the rate of reduction of cytochrome P-450 while nitrogen-containing compounds, such as aniline, cause a type II spectra and decelerate it. This observation may prove to be extremely important since the rate limiting step in microsomal drug oxidations is believed to be reduction of P-450 (5).

*Supported in part by USPHS Grant AM 10081 04 from the NIAMD and by USPHS Training Grant 5 T01 00010 from the NICHD.

[1] Type I difference spectra have absorption minima at about 420, 530 & 570nm and a broad maximum at about 385nm (3,4). Type II spectra have absorption maxima at about 430, 550 & 580nm and a minimum at about 390nm (3).

In adrenal mitochondria type I spectra are induced only by hydroxylatable steroids (6). Because several of the nitrogen-containing compounds inducing type II spectra, e.g., metyrapone, amphenone and aminoglutethimide, in adrenal mitochondria are potent inhibitors of steroid hydroxylation reactions as well, we have examined their effect on altering the rate of cytochrome P-450 reduction as reflected by the formation of the complex between reduced cytochrome P-450 and carbon monoxide (CO).

METHODS. Fresh bovine adrenal glands were extensively perfused through the adrenal artery with isotonic saline until the cortical tissue appeared a uniform tan color. From a 10% (w/v) homogenate the mitochondria were isolated by differential centrifugation (8). Subsequently, they were twice washed to remove endogenous electron donor substrate, adjusted to a protein concentration of 30-40mg/ml and kept at 4° until used. The oxygen uptake of these mitochondria was less than 0.1μM/min/mg protein, and in the absence of exogenous electron donor was not stimulated by added ADP. Difference and dual wavelength spectra were recorded using a Phoenix Dual Wavelength Scanning Spectrophotometer. Only those preparations in which hemoglobin could not be detected spectroscopically were used (9). Mitochondrial protein was determined by a biuret procedure (10).

RESULTS AND DISCUSSION. When added to aerobic adrenocortical mitochondria, metyrapone (7,11), amphenone, aminoglutethimide (11) and aniline induced type II difference spectra. Table 1 summarizes the location of the observed absorption maxima and minima for the three steroid hydroxylase inhibitors, and for aniline.

Figure 1 shows the kinetics of cytochrome P-450 reduction in the presence and absence of metyrapone. In the control experiment (Fig. 1A) after the aerobic, electron donor depleted mitochondria were gassed with CO, 10μM rotenone[2] was added. Addition of 5mM malate caused a rapid change in absorbance between 450

[2] In order to assess the spectral changes occurring during the reduction of cytochrome P-450, rotenone was added to prevent reduction of the conventional respiratory chain enzymes. Previous studies have established that rotenone has little effect on malate-supported 11β-hydroxylation (7).

Table 1

Comparison of the Absorption Maxima and Minima of Compounds Inducing Type II Spectral Changes With Adrenocortical Mitochondria

Compound	Conc.	Wavelength (nm) Maxima	Shoulder or small maximum	Minimum
Metyrapone	100μM	426 & 582	542	390
Amphenone	1mM	426 & 582	542	390
Amino-Glutethimide	1mM	426 & 582	542	392
Aniline	5mM	428 & 584	546	396

Difference spectra were obtained at room temperature with aerobic adrenocortical mitochondria suspended in a buffer solution consisting of 0.3M mannitol; 0.1mM disodium EDTA; 2.5mM phosphate buffer, pH 7.45; and 5mM HEPES (N-2-hydroxy-ethylpiperazine-N-2-ethane-sulfonic acid) buffer, pH 7.45 to a concentration of 2-3mg protein/ml.

and 490nm. Presumably, this absorbance difference is due to the progressive formation of the reduced P-450·CO complex which has an absorption maximum at 450nm. The reaction rate remained linear during the initial 5-10 seconds when the rate of absorbance change was most rapid. In calculating reaction velocities, measurements were taken only from the linear portion of the records, and the reported extinction coefficient of $91 mM^{-1} cm^{-1}$ was used (12). In this experiment (Fig. 1A) the rate of formation of the P-450·CO complex was 0.3nMoles/min/mg protein. When deoxycorticosterone (DOC) was added prior to malate, the rate increased to only 5.2nMoles/min/mg protein, a rate at least three to five times slower than the rate of malate-supported 11β-hydroxylation of DOC, according to Cammer and Estabrook[3] (14). As shown in 1B, the addition of 25μM metyrapone almost completely blocked the reduction of P-450 by malate.

[3]The discrepancy between the rate of P-450·CO complex formation in the presence of oxygen and the rate of 11β-hydroxylation has been previously noted (for discussion, see Cammer (15)).

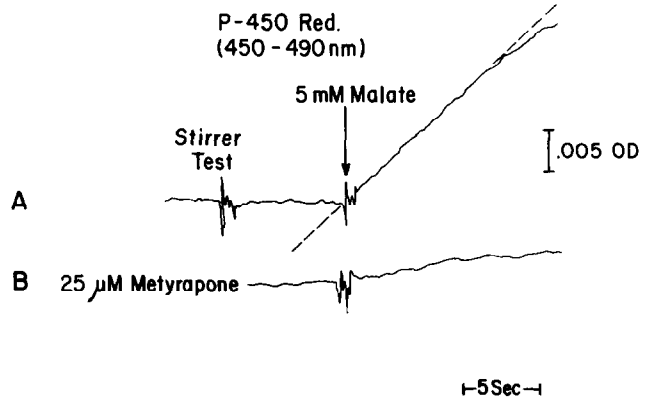

Fig. 1. Effect of metyrapone on the rate of malate-supported reduced cytochrome P-450·CO formation. The cuvette containing the aerobic adrenocortical mitochondria (5.6mg protein/ml) was diluted to 2.5ml with buffer solution as described in Table 1. The mixture was gassed for one minute with 100% CO, 10μM rotenone was added and the cuvette placed in the spectrophotometer (Dual Wavelength mode). In both records the resulting changes in absorbance (450-490nm) following the addition of 5mM malate were monitored. Record A is the control experiment. Record B shows the effect of adding 25μM metyrapone prior to the malate (Exp. BA-6824).

Although not shown in this figure, metyrapone also prevented the DOC-induced increase in this rate.

Comparable results were obtained with both 1mM amphenone and aminoglutethimide. Since removal of the primary amine group from aminoglutethimide is known to decrease its effectiveness as a steroid hydroxylase inhibitor (13), the experiments were repeated using 1mM glutethimide. It was much less effective than aminoglutethimide, causing only a 50-60% decrease in the initial, malate-supported rate of P-450 reduction as compared to the control value. Furthermore, the location of the absorption maxima and minimum of the glutethimide-induced difference spectrum was shifted to somewhat shorter wavelengths (max. at 422, 535 and 568nm, and a min. at 386nm) compared with those for aminoglutethimide (Table 1).

Because there is no easy way to be certain that the observed dual wavelength changes are due to an absorbance increase at the cytochrome P-450·CO peak (450nm) rather than to a decrease at the reference wavelength (490nm) the cyto-

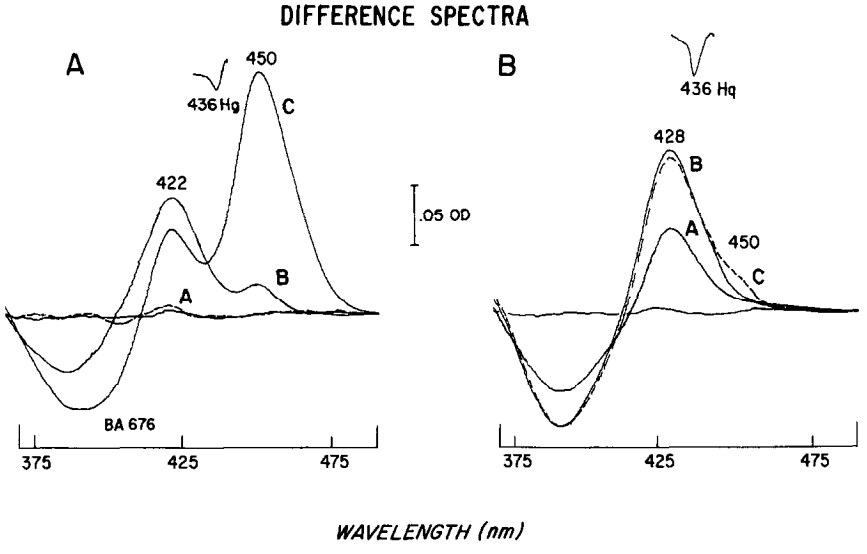

Fig. 2. (A) Difference spectra showing the effect of malate on the formation of reduced cytochrome P-450·CO. Aerobic adrenocortical mitochondria were suspended in the same buffer solution as described in Figure 1 at a concentration of 4.2mg protein/ml. After addition of 10μM rotenone the mitochondrial suspension was divided equally between two cuvettes and the baseline of equal light absorbance obtained. Following this the experimental cuvette was gassed with CO for one minute and the wavelength scanned again (curve A, dashed line). Curve B was recorded immediately on adding 5mM malate to the cuvette; curve C was recorded 5 minutes later. Prior to obtaining this spectrum both the experimental and reference cuvettes were oxygenated. The deflection at 436nm represents the mercury (Hg) blue line. Slit width, 0.2mm. (Exp. BA-676).

(B) Effect of metyrapone in preventing the malate-supported reduced cytochrome P-450·CO formation. All reaction conditions were the same as in Figure 2A as well as the concentration of mitochondria and rotenone. Gassing the experimental cuvette with CO caused no change in the baseline. Curve A was obtained after the addition of 25μM metyrapone to the experimental cuvette and an equal volume of ethanol to the reference cuvette. Curve B (solid line) was recorded immediately following 5mM malate addition to the experimental cuvette; curve C (dashed line), 15 minutes later. Oxygenation of both cuvettes was assured in each case.

---

chrome P-450 response was examined further by taking serial difference spectra of the Soret region following each addition. In Figure 2A, after recording the baseline of equal light absorbance the experimental cuvette was gassed with CO. This produced no appreciable change in the baseline (curve A, dashed line). Since only the reduced form of cytochrome P-450 can complex with CO, the absence of a peak implies that the P-450 in these mitochondria exists in the oxidized form. When malate was added to the experimental cuvette (curve B) in addition to

the peak at 422nm, a small peak at 450nm appeared immediately, signifying that some of the P-450 had been converted to the reduced form. With time the intensity of the 450nm peak gradually increased while that of the 422nm peak decreased (curve C).

When metyrapone was added to a fresh suspension of aerobic mitochondria treated with rotenone and CO, a peak appeared at 428nm (Fig. 2B, curve A). Although the subsequent addition of malate increased the intensity of this peak (curve B), no peak appeared at 450nm. Even after 15 minutes the only spectral evidence for the formation of the reduced P-450·CO complex was a small shoulder at 450nm. These difference spectra indicate that the spectral changes observed by the dual wavelength method at 450-490nm are due to changes in cytochrome P-450 at 450nm.

Type II spectral changes presumably reflect the binding of amines to oxidized cytochrome P-450. This implies that metyrapone, amphenone and aminoglutethimide express their inhibitory action by altering the kinetic properties of cytochrome P-450, rather than those of any other electron carrier in the mitochondrial steroid hydroxylase system. According to our view, steroid hydroxylation is inhibited because the P-450 inhibitor complex is reduced at a much slower rate than either free cytochrome P-450 or that complexed to DOC. Moreover, the finding that metyrapone decreases the rate of P-450 reduction even in the absence of DOC suggests that the mechanism of metyrapone inhibition of 11β-hydroxylation involves more than just competitively preventing the interaction of DOC with P-450 (14). Based on the spectral and kinetic changes observed, our data suggest that the effectiveness of the steroid hydroxylase inhibitor, aminoglutethimide, results from the presence of the primary amine. However, the failure of 5mM aniline to inhibit 11β-hydroxylation,[4] even though it induces a typical type II spectrum (Table I) when added to adrenocortical mitochondria, implies a requirement for a structure more complex than a hydro-

[4] Unpublished observation.

phobic amine. Furthermore, these data suggest that the spectral change (type II) induced by metyrapone, amphenone and aminoglutethimide may be unrelated to their inhibitory action.

We wish to thank Dr. J. J. Chart of Ciba Pharmaceutical Company, Summit, New Jersey, for supplying the metyrapone, amphenone, aminoglutethimide and glutethimide used in these experiments.

REFERENCES:

1. Gigon, P. L., Gram, T. E., and Gillette, J. R., Biochem. Biophys. Res. Commun. 31: 558 (1968).
2. Gigon, P. L., Gram, T. E., and Gillette, J. R., Mol. Pharmacol. 5: 109 (1969).
3. Remmer, H., Schenkman, J., Estabrook, R. W., Sesame, H., Gillete, S., Narasimhulu, S., Cooper, D. Y., and Rosenthal, O., Mol. Pharmacol. 2: 187 (1966).
4. Cooper, D. Y., Narasimhulu, S., Slade, A., Raich. W., Foroff, O., and Rosenthal, O., Life Sci. 4: 2109 (1965).
5. Imai, Y., and Sato, R., J. Biochem. (Tokyo) 62: 239 (1967).
6. Mitani, F., and Horie, S., J. Biochem. (Tokyo) 65: 269 (1969).
7. Harding, B. W., Bell, J. J., Wilson, L. D., and Oldham, S. B., in Functions of the Adrenal Cortex, ed. by K. McKerns, Vol. 2, p. 831, New York (1968).
8. Cammer, W., Cooper, D. Y., and Estabrook, R. W., in Functions of the Adrenal Cortex, ed. by K. McKerns, Vol. 2, p. 943, New York (1968).
9. Hosoya, T., and Morrison, M., Biochem. 6: 1021 (1967).
10. Szarkowska, L., and Klingenberg, M., Biochem. Z. 338: 674 (1963).
11. Whysner, J. A., and Harding, B. W., Fed. Proc. 27: 349 (1968). Abst.
12. Omura, T., and Sato, R., J. Biol. Chem. 239: 2379 (1964).
13. Cohen, M. P., Proc. Soc. Exp. Biol. and Med. 127: 1086 (1968).
14. Cammer, W., and Estabrook, R. W., Arch. Biochem. 122: 721 (1967).
15. Cammer, W., "Electron Transport and Energy Linked Reactions for Steroid Hydroxylation by Adrenal Cortex Mitochondria," (Ph.D. Thesis), #67-7826, University Microfilms, Inc., Ann Arbor, Michigan, p. 129-30 (1967).