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Ligand Modification of Corpus Luteum Mitochondrial Cytochrome P-450 Spectra and Cholesterol Monooxygenation: An Assay of Enzyme-Specific Inhibitors†

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ABSTRACT: Absorbance changes in the spectrum of cytochrome P-450 were related to the inhibition of [26-^{14}C]cholesterol oxidation to [^{14}C]isocaproate and pregnenolone in mitochondria from bovine corpus luteum produced by two types of ligands. Nitrogenous inhibitors, such as aminoglutethimide, elicit an absorption maximum at about 427 nm and a minimum at about 393 nm (type II), while steroidal inhibitors, such as (20*R*)-20-(*p*-tolyl)-5-pregnene-3β,20-diol (20-tolyl-pregnenediol), cause difference spectra with maximum at about 420 nm and minimum at about 390 nm (reverse type I). The magnitude of spectral change and the amount of inhibition of pregnenolone synthesis by aminoglutethimide are closely correlated at concentrations ranging from 5 to 750 μM and by the model steroid, 20-tolyl-pregnenediol, at concentrations from 0.5 to 25 μM. The responses are concentration dependent and linear over the range of effective concentrations. The concentrations of inhibitors for the half-maximal inhibition of pregnenolone biosynthesis are identical with the concentrations producing half-maximal spectral changes within experimental error. Displacement of substrate from cytochrome P-450 and/or stabilization of the redox potential subsequent to the ligation of heme iron is proposed as the specific mechanism of cholesterol side chain cleavage inhibition. Finally, together, the two procedures offer a sensitive, specific, and accurate means of screening inhibitors of the cholesterol side chain cleavage system.

Studies on the spectral properties of cytochrome P-450 (P-450) have shown that binding of substrates can be determined spectrophotometrically with ease and precision (Narasimhulu et al., 1965). The sites of interaction cannot yet be specified, but at least three types of induced spectral changes associated with the membrane-bound P-450 have been recognized (Schenkman et al., 1972).

The first type, type I, is caused by the addition of substrates to the oxidized form of P-450; it is characterized by an absorption maximum at about 390 nm and a minimum at 420 nm in difference spectrum. These ligands are associated with an EPR[1]-measurable conversion of the low-spin form of cytochrome P-450 to the high-spin form (Mitani and Horie, 1969). Conversely, the addition of certain reaction products causes formation of a peak at about 420 nm and a trough at 392 nm in the difference spectrum and is termed reverse type I (RT I). This spectral change has been attributed to the interaction of lipid soluble compounds at both the type I site and at another site on the cytochrome P-450 molecule (Schenkman et al., 1972). A third type of ligand, usually a nitrogenous base, interacts with cytochrome P-450 and causes a ferrihemochrome spectral change with an absorption maximum between 425 and 435 nm and a minimum at about 390 nm (type II). The type I and nitrogenous compounds are found to displace carbon monoxide from the reduced hemoprotein suggesting interaction with the heme (Symms and Juchau, 1973; Schenkman et al., 1967).

Gigon et al. (1969) with liver microsomes demonstrated that type I ligands usually increase the initial rate of reduced P-450 carbon monoxide complex formation, while type II compounds generally decrease it. Similarly, compounds of reverse type I class also decrease the rate of P-450 reduction. With bovine corpus luteum cytochrome P-450, their effectiveness correlates with their spectral binding affinity (McIntosh et al., 1973) but, with adrenocortical mitochondria containing a mixture of P-450 cytochromes, spectral effects of ligands are not consistent. The protein from the corpus luteum is a spectrally homogeneous protein (Užgiris et al., 1975; McIntosh et al., 1971).

The results reported here show that: (1) a good correlation exists between the intensity of ligand-induced spectral change and the amount of inhibition of pregnenolone[2] synthesis in

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[1] Abbreviations used: EPR, electron paramagnetic resonance; P-450$_{CL}$, corpus luteum mitochrondrial cytochrome P-450; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; Mann, mannitol.

[2] Trivial names used are: cholesterol, 5-cholesten-3β-ol; 20α-hydroxycholesterol, 5-cholesten-3β,20α-diol; pregnenolone, 3β-hydroxy-5-pregnen-20-one; 16α-chloropregnenolone, 16α-chloro-3β-hydroxy-5-pregnen-20-one.

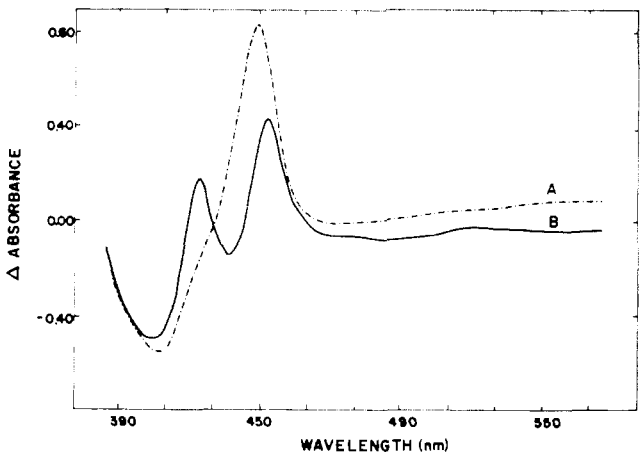

FIGURE 1: Carbon monoxide difference spectra of mitochondria and partially purified mitochondrial P-450 from bovine corpus luteum. (A) Dithionite-reduced partially purified P-450. Both the sample and reference cuvettes contained 1.0 mg of protein/ml of 0.1 M phosphate buffer, pH 7.0. The contents of the sample cuvette was saturated with CO, a few crystals of solid dithionite were added to both cuvettes, and the difference spectrum of the reduced cytochrome P-450-CO complex minus reduced cytochrome P-450 was recorded after 15 min. (B) Dithionite-reduced mitochondria. Both the sample and reference cuvettes contained a mitochondrial suspension (1.0 mg of protein/ml of Mann-Hepes buffer, pH 7.4). Subsequent treatment was similar to that described for A. Spectrum of mitochondria was recorded at a sensitivity twice that of P-450.

mitochondria from bovine corpus luteum; (2) the association applies to both type II and RT I ligands with the exception of compounds that are metabolized; (3) the ligands have equivalent effects on the cytochrome P-450 spectrum when intact mitochondria and purified preparations are compared. These findings make possible consideration of structure-activity relationships of the ligands, their mode of inhibition of P-450_{CL}, and the development of specific inhibitors of the cholesterol side chain cleavage enzyme (cholesterol monooxygenation).

Experimental Procedure

Preparation of Mitochondrial Fraction and Cytochrome P-450. Mitochondria from fresh bovine corpora lutea were prepared as described previously (McIntosh et al., 1971) with the following modifications. The isolation solution consisted of 10 mM Tris-HCl buffer, pH 7.8, containing 250 mM sucrose, 0.2 mM EDTA, and 1 mM sodium succinate. To remove lipids and hemoglobin, mitochondrial suspensions were strained through two layers of cotton gauze and were washed once by resedimenting from 0.154 M KCl. The final mitochondrial pellet was suspended in a buffer consisting of 300 mM mannitol, 5 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 0.2 mM EDTA, 2.5 mM potassium phosphate, pH 7.4 (Mann-Hepes buffer). The P-450_{CL} was purified according to a modified procedure of Mitani and Horie (1969) as reported by McIntosh et al. (1973). This procedure involves solubilization of hemoprotein with cholate and chromatography on Sephadex G-25 followed by an ammonium sulfate fractionation of P-450-containing column fractions. The cytochrome preparation had appreciably higher P-450 content (0.93 nmol/mg of protein) than that of the original mitochondrial fraction (0.22 nmol/mg of protein) (Figure 1). No other cytochromes could be demonstrated in the preparation, but it contained approximately 14 μg of cholesterol per mg of protein.

Difference Spectrometry. Difference spectra were recorded at room temperature in a Fisher FRL-1000 dual wavelength scanning spectrophotometer as described previously (Užgiris et al., 1975). The spectral changes caused by the addition of various ligands were recorded repetitively until the absorbance change in the Soret region was stabilized. The amount of spectral change (ΔA) was calculated as the difference in absorbance between peak and trough. The propylene glycol in which spectral ligands were added to the mitochondrial suspensions never exceeded 3% by volume and had no significant effect on the observed ΔA or enzymatic rate of cholesterol monooxygenation. Since the P-450 concentrations varied in different preparations, ΔA was usually calculated on a molar basis and is plotted as a function of ligand concentration. The half-saturation intercept of such a plot corresponds to the apparent dissociation constant (K_s) and can be determined graphically.

The coefficient of variation (Snedecor and Cochran, 1967) of the aminoglutethimide-induced spectral changes ascertained with the same mitochondrial preparation was 0.1 at aminoglutethimide concentration of 50 μM and 0.012 at 500 μM. However, when fresh mitochondria were tested at different times, the coefficient was 0.2 (n = 4). In contrast to the observed variation in the intensity of spectral change, the location of maxima and minima remained constant.

Cholesterol Monooxygenase Activity Assay. Enzyme activity was determined by a modification of the method described by Hochberg et al. (1974) which measures [^{14}C]isocaproate released from labeled cholesterol. Mitochondria were incubated at 37 °C in an assay mixture containing 1 mg of mitochondrial protein, 200 mM mannitol, 4.5 mM Hepes, 0.1 mM EDTA, 2.3 mM potassium phosphate, 11 mM $CaCl_2$, 4.5 mM NADPH, 0.03% bovine serum albumin, pH 7.4, in a final 1.5-ml volume (Užgiris et al., 1971).

Inhibitors were added in 25 μl of propylene glycol and an identical volume of the solvent was added to the control incubation mixture. Complete assay mixtures with added inhibitors were preincubated for 15 min at 0 °C before transferring to 37 °C for 2 min. The reaction was started with the addition of [26-^{14}C]cholesterol, 10^6 dpm, suspended with 20 μg of Tween 80 in 0.1 ml of Mann-Hepes buffer. Samples of 0.2 ml were withdrawn at 3-min intervals for 15 min. [^{14}C]Isocaproate was isolated by filtration through alumina. An internal standard of ^3H_2O (330 000 dpm/incubation) was used to correct for losses. The radioactivity associated with isocaproate was plotted as a function of time. The enzyme rate in pmol of cholesterol cleaved min^{-1} (mg of protein)$^{-1}$ was calculated by the method of least-squares from the initial linear phase of the plotted curve.

The coefficient of variation for the reaction rates within experiments was 0.035. It increased to 0.091 when rates were compared among different mitochondrial preparations.

To validate the [^{14}C]isocaproate assay procedure, effects of inhibitors on the cholesterol side chain cleavage assays were compared at identical concentrations. No significant differences were seen between the assay based on [^{14}C]isocaproate quantitation and the one based on the measurement of steroid products (Užgiris et al., 1971; McIntosh et al., 1971). In these comparisons the extent of inhibition by 20α-hydroxycholesterol, 17β-hydroxy-17α-methylandrost-4-eno[3,2-c]pyrazole and aminoglutethimide was identical within experimental error. The [^{14}C]isocaporate-based method, however, is considerably more rapid and reliable.

In some experiments the inhibition of cholesterol monooxygenase activity was determined by plotting percent inhibition of the control rate as a function of the inhibitor concentration. Under standard conditions, in such a plot the in-

tercept at 50% inhibition corresponds to I_{50} or the concentration of the inhibitor required to reduce the enzyme rate by half.

Chemical Procedures. The concentration of cytochrome P-450 and P-420 was determined by the method of Omura and Sato (1964). Protein concentrations were determined by the method of Lowry et al. (1951) or the biuret procedure of Szarkowska and Klingenberg (1963) using bovine serum albumin as a standard. Concentrations of aminoglutethimide and Δ^5-3β-hydroxysteroids were measured according to Douglas and Nicholls (1965) and Oertel and Eik-Nes (1959), respectively.

Chemicals and Enzymes. [26-^{14}C]Cholesterol (54 mCi/mmol), [7α-^{3}H]cholesterol (24 Ci/mmol), and ^3H_2O (100 μCi/mmol) were obtained from New England Nuclear. NADPH, bovine serum albumin, and glucose-6-phosphate dehydrogenase were purchased from Sigma. The racemic mixture of aminoglutethimide, *dl*-3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione, 2-methyl-1,2-bis(3-pyridyl)-1-propanone (metyrapone), 1-(*p*-methoxyphenyl)-1-(3-pyridyl)-2-methylpropanol (Su 7550), *l*-aminoglutethimide, *l*-aminoglutethimide tartrate, *d*-aminoglutethimide tartrate, *N*-aminoglutethimide, 3-ethyl-3-phenyl-1-aminopiperidine-2,6-dione were gifts of Drs. J. J. Chart and B. Steinetz, CIBA-GEIGY Pharmaceutical Co., Summit, N.J. Other aminoglutethimide analogues, 3-ethyl-3-(4-aminophenyl)-1-methyl-piperidine-2,6-dione (*N*-methylaminoglutethimide), 3-ethyl-3-(4-aminophenyl)-1-benzylpiperidine-2,6-dione (*N*-benzylaminoglutethimide), and 3-ethyl-3-(4-aminophenyl)-3,4-dehydropiperidine-2,6-dione (ethylglutaconimide), were generous gifts of Dr. E. C. De Renzo, Lederle Laboratories. 17β-Hydroxy-17α-methylandrost-4-eno[3,2-*c*]pyrazole was a product of Winthrop Chemicals. (20*R*)-20-(*p*-Tolyl)-5-pregnene-3β,20-diol (20-tolyl-pregnenediol) was generously given by Drs. R. G. Hochberg and S. Lieberman, Columbia University. 4-Phenylimidazole and 1-benzylimidazole were purchased from Aldrich Chemicals. All other steroids were obtained from Steraloids, Inc.

Results

Enzyme Assay. To obtain reasonable zero-order kinetics for cholesterol side chain cleavage with intact mitochondria, the assay conditions were evaluated to achieve linear initial rates of product formation. The rates were linear with respect to mitochondrial protein concentration within a range of 0.5–1.5 mg of protein and decreased when the protein concentration exceeded 2 mg/ml. Maximal rates were observed at 4.5 mM NADPH in the presence of a swelling agent (11 mM $CaCl_2$) and after preincubation for 15 min at 0 °C. The pH optimum of the reaction was 7.0 at 37 °C over a range of 6.5 to 7.9. The rate was linear and reproducible during the initial 10 min of incubation and usually decreased after 10 to 15 min. Four samples obtained at 3-min intervals, including the zero time, sufficed for calculation. With many inhibitors, the reaction rate remained linear up to 30 min.

Ligand-Induced Spectral Changes of Cytochrome P-450 in the Mitochondria from Bovine Corpus Luteum. Two types of difference spectra were recorded on addition of selected steroid and aromatic N-heterocyclic ligands to a mitochondrial suspension with the P-450 in the oxidized state. A reverse type I spectral change (Figure 2, curve A) was recorded when 20-tolyl-pregnenediol, pregnenolone, or progesterone was added to the sample cuvette. The Soret γ maximum at about 420 nm and the minimum at about 390 nm are thought to represent ligand binding to the apoprotein, which results in a

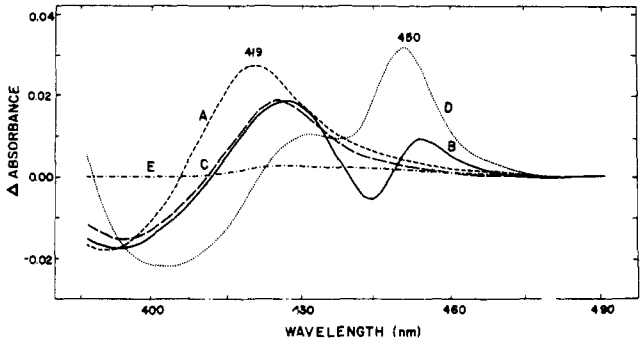

FIGURE 2: Difference spectra induced by the addition of aminoglutethimide, 20-tolyl-pregnenediol, and CO to aerobic or $Na_2S_2O_4$-reduced bovine corpus luteum mitochondria. Mitochondria were suspended in Mann–Hepes buffer to 1.3 mg of protein/ml. For reaction conditions, see Experimental Procedure. Spectra were recorded with aerobic mitochondria and (A) 25 μM 20-tolyl-pregnenediol, (C) 250 μM aminoglutethimide, and (E) CO added to sample cuvettes. Difference spectrum D was recorded after reducing E cuvettes with $Na_2S_2O_4$ for 15 min, and B was recorded after bubbling CO into the sample cuvette and adding $Na_2S_2O_4$ to both cuvettes of A.

conformational change followed presumably by an altered orientation of the axial ligands of the heme (Tsai et al., 1970). Addition of dithionite to 20-tolyl-pregnenediol-containing mitochondria resulted in 30% decrease in ΔA between 419 and 389 nm. On subsequent exposure of the sample cuvette to carbon monoxide, only a small peak appeared at 450 nm indicative of the reduced P-450-CO adduct (Figure 2, curve B). The decrease of ΔA at 450 nm by 70% in presence of 20-tolyl-pregnenediol when compared with ΔA of an identical preparation without it (Figure 2, curve D) may indicate competition between CO and 20-tolyl-pregnenediol.

Aminoglutethimide, a type II compound, induced spectral changes in the corpus luteum mitochondria with the absorption maximum at 427 nm in the Soret region (Figure 2, curve C). The aminoglutethimide induced spectral change in the Soret region had an identical λ_{max} as that observed in adrenocortical mitochondria (McIntosh and Salhanick, 1969), even though adrenocortical mitochondrial P-450 preparations contain at least three P-450-linked mixed-function oxidases.

As previously reported (McIntosh et al., 1973), cholesterol was without effect on the spectra of mitochondrial preparations even though a type I spectrum might be expected for substrates of cholesterol monooxygenase. Moreover, neither incubation of mitochondria for up to 1.5 h under conditions suitable for steroid synthesis nor partition of the particles between buffer and diethyl ether or isooctane sufficiently reduced the cholesterol concentration at the substrate binding site to yield type I spectrum. Both the RT I spectral change produced by 20-tolyl-pregnenediol or 20α-hydroxycholesterol and the type II spectral change of aminoglutethimide reported previously (Užgiris et al., 1975) showed concentration dependence.

Time Course of Ligand-Induced Spectral Change of Membrane-Bound Mitochondrial Cytochrome P-450. The intensity of spectral change obtained on addition of a ligand was found to depend on: (1) the affinity of the ligand; (2) the time interval after ligand addition; (3) the specific activity of the enzyme preparation; and (4) the concentration of the ligand. The time interval required to obtain maximal ΔA after addition of a ligand to the mitochondrial suspension or a partially purified P-450 preparation varied with the nature of the ligand, but in most cases it was obtained within 10 min (Figure 3).

The maximal ΔA remained constant for up to 30 min with

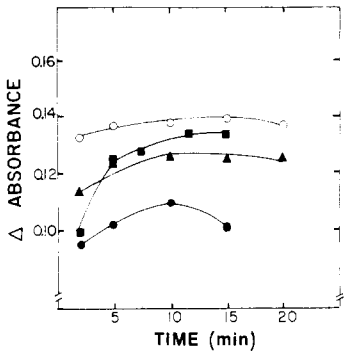

FIGURE 3: Kinetics of spectral change induction. Mitochondria were suspended and ligands were added as described in the legend to Figure 2. Spectra were recorded repeatedly from 506 to 380 nm at indicated time intervals. The absorbance difference between peak and trough was calculated per nmol of P-450: (○) 500 μM aminoglutethimide (λ_{max} 427 nm; λ_{min} 393 nm); (■) 5 μM 20-tolyl-pregnenediol (λ_{max} 419 nm; λ_{min} 389 nm); (●) 250 μM pregnenolone (λ_{max} 420 nm; λ_{min} 390 nm); (▲) 75 μM 16α-chloropregnenolone (λ_{max} 420 nm; λ_{min} 390 nm).

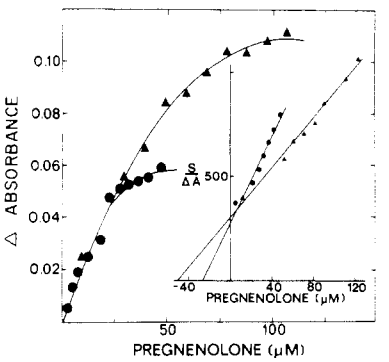

FIGURE 4: Effect of pregnenolone concentration on the difference spectra of mitochondria and of partially purified mitochondrial P-450. Mitochondria (▲) were suspended in Mann–Hepes buffer, pH 7.4, to contain 0.25 μM P-450 (1.2 mg of protein/ml). The partially purified cytochrome P-450 (●) was diluted to 1.5 μM (1.6 mg of protein/ml) concentration in 0.1 M phosphate buffer, pH 7.0. Difference spectra were recorded immediately following successive additions of pregnenolone in propylene glycol to the sample cuvette and propylene glycol to the reference cuvette. The absorbance difference between λ_{max} at 420 nm and λ_{min} at 390 nm was calculated *per nmol of P-450*. The inset shows the data in reciprocal plot. The apparent K_s values for mitochondria and the partially purified P-450 were 50 and 27 μM, respectively.

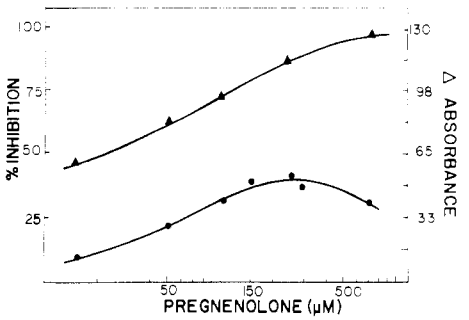

FIGURE 5: Relationship between absorbance increment and inhibition of cholesterol monooxygenation obtained with pregnenolone (reverse type I). Mitochondria from corpora lutea were incubated with 15 to 750 μM pregnenolone. Difference spectra were recorded in parallel experiments 7.5 min after the addition of pregnenolone as described in the legend to Figure 4. The absorbance difference (▲) between peak and trough was calculated per μmol of P-450. Control rate was 2.5 pmol of cholesterol cleaved min^{-1} (mg of protein)$^{-1}$. (●) Percent inhibition of control rate of cholesterol cleavage. The half saturation intercept of ΔA corresponds to K_s value of 19 μM.

all substances tested except pregnenolone. In an experiment designed to measure the time-dependent decay of the spectral change elicited upon the addition of pregnenolone to 15 μM, the ΔA recorded after 15 min was only 47% of that observed after 3 min of incubation. We have reported previously on the presence of an active 3β-hydroxysteroid dehydrogenase in mitochondria from corpus luteum (McIntosh et al., 1971; Užgiris et al., 1971). Thus, a transient spectral change is consistent with the assumption of a rapid transformation of pregnenolone to progesterone in the presence of endogenous pyridine nucleotides.

Binding Specificity of Corpus Luteum Mitochondrial Cytochrome P-450. Intact mitochondria and a partially purified preparation were compared for their spectral response to pregnenolone, a typical RT I ligand. The spectral traces in intact mitochondria and solubilized P-450 were similar both in shape and location of maxima and minima. For equivalent concentrations of P-450, the magnitude of ΔA calculated per nmol of P-450$_{CL}$, at spectral saturation level, was greater for the intact mitochondria than for the purified P-450$_{CL}$ (Figure 4). Consequently, the apparent K_s for the partially purified P-450$_{CL}$ was 27 μM, but for mitochondria it was 50 μM. It should be emphasized that this difference was not noted for 20α-hydroxycholesterol (Užgiris et al., 1975).

The disparity between these two preparations might be due to the cholate which is presumed to be present in our partially purified P-450$_{CL}$. Although sodium cholate by itself, in concentrations of up to 1.8 mM, failed to induce any spectral changes in P-450-containing mitochondrial membranes, the possibility that sodium cholate might interfere with the production of the RT I spectral change in the solubilized P-450 either by occupying the cholesterol binding sites or by modifying the conformation of the apoprotein can not be excluded. The development of a type I spectral change has been shown to be inhibited by nonionic detergent Triton N-101 (Bell et al., 1973), and a facile conversion of P-450 to P-420 takes place on exposure of corpus luteum mitochondria to larger concentrations (1.0–2.5 mg/ml) of sodium cholate (Užgiris et al., 1975). These observations also indicate that pregnenolone induced ΔA in mitochondria from corpus luteum is attributable to ligation of P-450 but not other hemoproteins since the partially purified P-450$_{CL}$ was devoid of other cytochromes.

Relationship between Reverse Type I Spectral Change and Cholesterol Monooxygenase Inhibition. The inhibition of cholesterol cleavage by pregnenolone has been long known (Raggatt and Whitehouse, 1966), but the mechanism was not clear. We, therefore, examined the effects of a wide range of concentrations of pregnenolone on the magnitude of the RT I spectrum and cholesterol monooxygenation in luteal mitochondria. A maximal spectral change was observed between 5 and 15 min after an addition of pregnenolone to mitochondria. The inhibition of cholesterol cleavage expressed as percent inhibition of control rate and ΔA per μmol of P-450 are plotted as a function of pregnenolone concentration (Figure 5). Maximal spectral change was established at the plateau of the response and was set to equal 100% inhibition. In such a plot a lack of parallelism between the two assay curves might suggest the presence of additional binding sites or inactivation of the inhibitor, but this was observed to occur only with pregnenolone. Both the percent inhibition and ΔA show concentration dependence. Half-maximal spectral saturation was obtained at about 20 μM pregnenolone. Even at concentrations producing almost complete spectral saturation, the observed inhibition did not exceed 50%. The enzyme rate in these ex-

periments was determined from the slope of the activity curve during the initial 15 min of incubation and the rate of cholesterol monooxygenase increased steadily after that interval. Thus, the increase of the enzyme rate with time and the associated rapid decrease of spectral binding indicate that pregnenolone is rapidly transformed into a less active substance. This may explain the apparent lack of an effect of pregnenolone on the initial fast phase of the P-450$_{CL}$ reduction (McIntosh et al., 1973).

On the other hand, the S-shaped RT I spectral response curve obtained with 16α-chloropregnenolone and plotted as for pregnenolone was concentration dependent and steeper than that of pregnenolone (Figure 6a, curve D). In addition, the reaction rate slopes were linear and ΔA did not decrease with time indicating that the enzyme-ligand complex is stable. We infer from these data the 16α-chloro substituent decreases the A,B ring oxidation, although it is possible that the substituent enhances binding to P-450 directly. The concentration of 16α-chloropregnenolone required to reduce the control rate of cholesterol monooxygenase by 50% (23 μM) was identical with the concentration required for an equivalent amount of spectral change (23 μM).

The *p*-tolyl-substituted pregnenolone derivative elicited the half-maximal RT I spectral change at 0.8 μM which is markedly lower than 16α-chloropregnenolone (23 μM) (Figure 6a, curve B). It inhibited [26-^{14}C]cholesterol cleavage half-maximally at an equally low concentration (0.6 μM), although it is known that in tracer concentrations the 20-tolyl-pregnenediol itself is converted to pregnenolone and progesterone in 25% yield (Hochberg et al., 1972). Both assay curves are considerably steeper than those for 16α-chloropregnenolone.

Neither pregnenolone nor 16α-chloropregnenolone displaced CO from reduced P-450$_{CL}$. The 20-tolyl-pregnenediol, however, inhibited the formation of the reduced P-450–CO complex (Figure 2, curve B), but failed to form a hemochromogen itself with the reduced P-450$_{CL}$.

Relationship between Aminoglutethimide-Induced Type II Spectrum and Cholesterol Monooxygenase Inhibition. The relationship between the type II spectral change induced by aminoglutethimide and its inhibition of mixed-function oxidation is shown in Figure 6b. The half-maximal inhibition of the enzyme rate occurs at approximately 60 μM. At 750 μM, or near the solubility limit of aminoglutethimide, spectral change approaches a plateau and the rate of cholesterol oxidation is inhibited to 7% of the control rate. As with pregnenolone and 16α-chloropregnenolone, the binding of aminoglutethimide is characterized by an S-shaped curve. This type of spectral saturation curve appears to be a characteristic of all P-450 enzymes, including the highly purified *Pseudomonas putida* protein (Gunsalus and Sligar, 1976) and is indicative of first-order rate processes.

The aminoglutethimide-induced spectral alteration developed rapidly, persisted for over 30 min, and was independent of the valency state of iron in cytochrome P-450$_{CL}$. The aminoglutethimide spectrum is not affected by sodium dithionite treatment, unlike the metyrapone-induced spectrum of liver microsomal P-450 (Hildebrandt, 1972), which shows a bathochromic shift of the peak to 450 nm after reduction with sodium dithionite. Furthermore, aminoglutethimide had no effect on the magnitude of the final reduced P-450–CO spectral change. Metyrapone, however, produced the expected shift to 450 nm with dithionite reduced corpus luteum P-450 (data not shown) just as it does with liver microsomal or adrenocortical mitochondrial P-450 (Schleyer et al., 1972). This indicates that aminoglutethimide binds with a lesser affinity to P-450$_{CL}$ than 20-tolyl-pregnenediol and in a different manner than metyrapone since no spectral coordination between reduced P-450 and the primary amine or imide of the heterocycle could be shown.

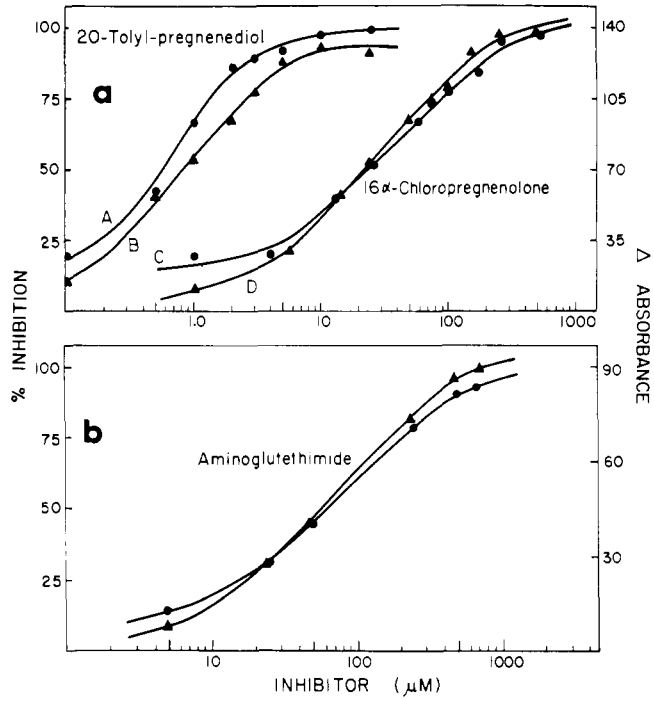

FIGURE 6: (a) Relationship between absorbance increment and inhibition of cholesterol monooxygenase obtained with 20(*R*)-20-(*p*-tolyl)-5-pregnene-3β,20-diol (A and B) and 16α-chloropregnenolone (C and D) (both reverse type I). Other conditions were as described in the legend to Figure 5 except that difference spectra were recorded 12 min after the addition of the ligand. Control rates were 5.8 and 7.9 pmol of cholesterol cleaved min^{-1} (mg of protein)$^{-1}$, for inhibition experiments (●) with 20-tolyl-pregnenediol (A) and 16α-chloropregnenolone (C), respectively. The absorbance difference, ΔA per μmol of P-450 (▲), was calculated between λ_{max} at 419 nm and λ_{min} at 389 nm for 20-tolyl-pregnenediol (B) and λ_{max} at 420 nm and λ_{min} at 390 nm for 16α-chloropregnenolone (D). Corresponding values of I_{50} and K_s were 0.6 and 0.8 μM for 20-tolyl-pregnenediol but both 23 μM for 16α-chloropregnenolone. (b) Relationship between absorbance increment (▲) and inhibition of cholesterol monooxygenase (●) obtained with 5 to 750 μM aminoglutethimide (type II). Conditions were as described in the legend to Figure 5, except that difference spectra were recorded 12 min after the addition of aminoglutethimide. The ΔA between λ_{max} at 427 nm and λ_{min} at 393 nm was calculated per μmol of P-450. Control rate was 18 pmol of cholesterol cleaved min^{-1} (mg of protein)$^{-1}$. The values of I_{50} and K_s were 60 and 55 μM, respectively.

Relationship between Type II Spectral Change and Cholesterol Monooxygenase Inhibition. Certain nitrogenous compounds are known to inhibit steroidogenesis in adrenal cortex (Kahnt and Neher, 1966). Some of these, e.g., metyrapone and aminoglutethimide, elicit changes in P-450 spectra; therefore, we attempted to determine if the correlation between the spectral change and the inhibition is a common property of all of these compounds. Fourteen nitrogenous heterocylic compounds were examined by both assay procedures at only 50 and 500 μM concentration levels. Figure 7 depicts plots where the extent of inhibition is plotted as a function of type II spectral change shown as ΔA between λ_{max} and λ_{min} calculated per μmol of P-450. This more expedient analysis is different from previous plots in that single ΔA measures for different compounds are compared without adjustment for the maximal response. At 50 μM inhibitor concentration the product-moment correlation coefficient between binding and

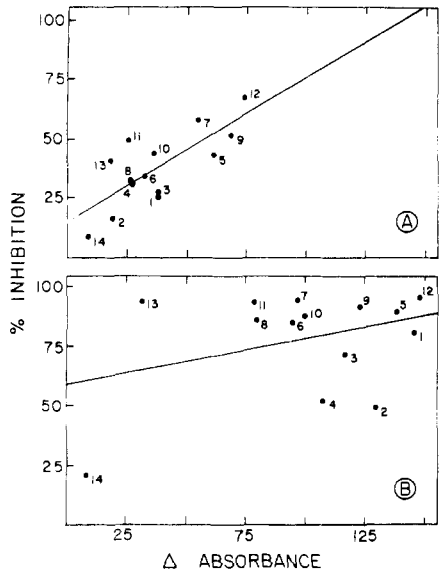

FIGURE 7: Correlation scattergrams of ligand-induced type II spectral changes and inhibition of cholesterol monooxygenase in mitochondria from corpus luteum. Ligands were tested and correlated at 50 μM (A) and 500 μM (B) concentrations in the enzyme and the spectral assays. For the reaction conditions, see Experimental Procedure. Cholesterol monooxygenase inhibition for each ligand is plotted as a function of absorbance difference between peak and trough in the Soret region and calculated per μmol of P-450. Ligands that were tested in both procedures are: (1) 1-benzylimidazole; (2) 4-phenylimidazole; (3) metyrapone; (4) Su 7550; (5) aminoglutethimide; (6) *l*-aminoglutethimide; (7) *d*-aminoglutethimide; (8) *l*-aminoglutethimide tartrate; (9) *d*-aminoglutethimide tartrate; (10) 3-methylaminoglutethimide; (11) *N*-methylaminoglutethimide; (12) *N*-aminoglutethimide; (13) *N*-benzylaminoglutethimide; (14) ethylglutaconimide. Correlation coefficient, r, at 50 μM (A) was 0.77 and r at 500 μM (B) was 0.36.

inhibition was 0.77, and at 500 μM the coefficient was 0.36. At 500 μM the inhibition showed a trend to cluster around 12% of control rate. Only at 50 μM was the correlation coefficient significantly different from zero ($p < 0.001$). Analysis of variance on these data revealed a significant overall concentration effect ($F = 37.52$; df 1, 26; $p < 0.01$), but the difference between spectral binding and enzyme inhibition was not significant at either concentration level.

The observations indicate that under these experimental conditions there is a good relationship between spectral binding of a nitrogenous ligand and the inhibition of the P-450 catalyzed cholesterol monooxygenation. Furthermore, nitrogenous compounds differing considerably in structure show the correlation between spectral binding and enzymatic inhibition.

Discussion

In this paper we describe a rapid and specific two-phase assay for screening inhibitors of the cholesterol side-chain cleavage enzyme system. The assay is based on the correlation of ligand induced spectral changes and the inhibition of mitochondrial P-450 which oxidizes [26-^{14}C]cholesterol to [^{14}C]isocaproate and pregnenolone. The conditions for validation of such an assay were met by showing that (1) the amount of induced RT I or type II spectral change parallels the amount of enzyme inhibition; (2) the responses are concentration dependent and linear over an effective range of concentrations; and (3) the concentration of an inhibitor for the half-maximal inhibition of pregnenolone biosynthesis (I_{50}) is identical with the concentration producing the half-maximal spectral change (K_s) within the experimental error (see Figure 6a and b for results with typical RT I and type II ligands, respectively).

Enzyme Assay. The measured [^{14}C]isocaproate was found to be a valid index of cholesterol side chain cleavage in luteal mitochondria and was more accurate than the measurement of the steroid products, pregnenolone and progesterone. To obtain reasonable zero-order kinetics, however, certain restrictions on mitochondrial protein concentrations and time of sampling of incubation media are necessary. Since certain inhibitors may be unstable (e.g., pregnenolone which is rapidly converted to progesterone with marked loss of affinity for the enzyme), calculation of inhibition is based upon initial enzyme rates. More importantly, the enzyme rate decreases after 10–15 min. A temperature-dependent enzyme instability has been postulated as one possible cause for the decreasing enzyme rate (Hochberg et al., 1974), and, alternatively, Arthur and Boyd (1974) suggested that the decrease may be due primarily to the depletion of the readily available [^{14}C]cholesterol to the enzyme complex. The latter explanation is unlikely since only 8–10% of the added radioactive substrate is utilized in a 30-min incubation with mitochondria from corpus luteum. On the other hand, a slow decrease in enzyme activity was observed even when mitochondrial suspensions were kept on ice.

Spectral Assay. The spectral effects of various ligands on heterogenous cytochromes P-450 have been described many times, but observations as well as interpretations have been conflicting. One reason for this is the multiplicity of cytochromes P-450 with different substrate specificities in most steroidogenic or hepatic subcellular particles. For example, deoxycorticosterone added to adrenocortical mitochondria causes a type I spectrum as it associates with the 11β-steroid-hydroxylating P-450, but a RT I spectrum is generated with the cholesterol side chain cleavage P-450. Both spectral contributions overlap and yield results dependent on the proportion of the two enzymes and the individual affinities of the ligand. For studying enzyme–substrate binding, therefore, it is mandatory to have a preparation containing a single P-450; this appears to be the case for mitochondria from the bovine corpus luteum.

Correlation of Spectral Binding and Enzyme Inhibition. In our preparations type I spectra were not produced by the addition of cholesterol to the membrane-bound P-450, although several other laboratories have demonstrated such spectra with cholesterol-depleted cytochrome P-450 from the adrenal cortex (Burstein et al., 1972; Harding et al., 1971). This implies that the substrate binding site of mitochondrial P-450 is saturated with endogenous cholesterol and is consistent with the theory that the RT I spectrum results from the displacement of bound substrate cholesterol (Schenkman et al., 1972). The partially purified preparation of P-450$_{cl}$ used in our studies did demonstrate a small increase in oxygen consumption with the addition of cholesterol to the reconstituted system.[3] Presumably, even that preparation did not have the substrate binding site sufficiently depleted of cholesterol to elicit the expected type I spectrum. Thus, current theory would propose that the spectral changes which are elicited with pregnenolone, 16α-chloropregnenolone, and 20-tolyl-pregnenediol result from such a displacement. A substance of special interest is 20α-hydroxycholesterol which can act as a substrate, elicit a type I spectrum with acetone extracted mitochondria or a RT I spectrum with native mitochondria, and inhibit [^{14}C]cholesterol cleavage. Furthermore, 20α-hy-

[3] F. Mitani, V. I. Užgiris, N. McIntosh, and H. A. Salhanick, unpublished observations.

droxycholesterol inhibited reduction of partially purified P-450$_{CL}$ (McIntosh et al., 1973). Since RT I spectra have been reported to occur with P-450 preparations of liver microsomes from which endogenous substrate has been removed by solvent extraction (Vore et al., 1974), a corollary to the displacement theory must provide for the apparent direct effect of a ligand on the cytochrome.

Regardless of the mechanism of inhibition all of the tested RT I ligands with the possible exception of pregnenolone showed a good correlation between cholesterol cleavage and spectral change.

With type II compounds the correlation between the P-450 binding and inhibition was even better. Thus, for the model compound, aminoglutethimide, there is a good parallelism between spectral change and inhibition over a wide concentration range. The close relationship between inhibition and binding to P-450 exists for a number of nitrogen-containing aromatic bases and is not a unique property of the aminoglutethimide–P-450 interaction (Figure 7). The calculated correlation coefficient of 0.77 at 50 μM is sufficiently high to indicate that the observed inhibition is essentially due to binding of a base to P-450$_{CL}$ despite the inherent experimental errors of procedures performed with different mitochondrial preparations.

Molecular Implications. The nature of the enzyme active center may be further studied by observing the effects of ligands on the CO-ligated P-450 spectrum. Both CO and O_2 are accepted to be interchangeable in their mode of binding to P-450. Displacement of CO from the heme iron by type I ligands has been demonstrated with placental P-450 (Symms and Juchau, 1973) and by certain type II ligands with adrenal mitochondrial P-450 (Schleyer et al., 1972). It is now evident that such a displacement also occurs upon addition of a RT I ligand, 20-tolyl-pregnenediol (Figure 2). These observations imply that inhibition may result from interference with the binding either of cholesterol or of oxygen required for the monooxygenase reaction.

The spectral change produced by metyrapone (type II) with the P-450$_{CL}$ in the reduced state was found to be identical with that observed with adrenocortical (Schleyer et al., 1972) and liver microsomal P-450 (Hildebrandt, 1972). The hemochromogen-like spectrum is characterized by an absorbance maximum at around 446 nm. This indicates that metyrapone coordinates to the heme iron of P-450$_{CL}$ and may interfere with O_2 activation. On the other hand, aminoglutethimide does not produce a hemochromogen-like spectrum with the P-450$_{CL}$ and thus does not compete with O_2 for the heme iron. It most likely competes for the substrate site.

We propose the following reaction models for cholesterol side chain cleavage inhibition. Type I compounds such as cholesterol bind to a substrate site which may include amino acid residues of the apoprotein and possibly portions of the porphyrin ring structure. Interaction between steroid and metalloporphyrin has been demonstrated by Hill et al. (1973), but they proposed a plane-to-plane type of interaction. Specific RT I inhibitors resemble cholesterol and would either overlap or cover the active center. Depending on the affinity, the substrate would be displaced, O_2 activation curtailed, and even cleavage of an appropriately oriented portion of inhibitor itself might occur with some of these substances (Hochberg et al., 1972).

The mechanism of inhibition and the site of binding of type II ligands is somewhat different. The binding site must be similar to that discussed above for the steroid inhibitors and would overlap the substrate binding site because aminoglutethimide is a competitive inhibitor of cholesterol cleavage (Cohen, 1968). In addition, the aromatic ring of an inhibitor may participate in plane-to-plane interaction with the porphyrin as postulated by Nagata et al. (1976). Alternatively, aromatic bases with pyridyl, nitrile, or amine groups have considerable affinity for metalloporphyrins (Lowe, M. B., and Phillips, J. N., cited by Falk, 1964), stabilize redox potentials (Martell and Calvin, 1953), and could well determine the extent of interaction between an inhibitor and the heme iron of P-450$_{CL}$. One example is aminoglutethimide which inhibits several P-450-catalyzed hydroxylations but has a high degree of specificity for cholesterol-cleaving P-450. Although amines are weaker heme ligands than pyridyl or nitrile substituted ligands, the para amine in aminoglutethimide is essential for its specificity (McIntosh and Salhanick, 1969). Another aspect of its specificity is related to the 2,5-piperidinedione portion of the molecule.

The study of P-450–steroid complexes may provide new insights for understanding the enzymatic mechanism of cholesterol monooxygenation. The information on binding is important for the development of specific inhibitors of the P-450-catalyzed reactions because through this approach specific designs for such molecules may be proposed. Further information may be derived from studies on the stoichiometry of the P-450–ligand interaction site; however, this requires highly purified enzyme. Current efforts are directed toward these goals.

Acknowledgment

Authors are grateful to Drs. E. N. McIntosh and N. H. Sloane for many discussions in the course of the research and helpful suggestions in the preparation of this paper.

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Effect of Specific Trifluoroacetylation of Individual Cytochrome *c* Lysines on the Reaction with Cytochrome Oxidase†

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ABSTRACT: We have prepared three different cytochrome *c* derivatives, each containing a single specifically trifluoroacetylated lysine at residues 13, 55, and 99, respectively. The only modification that affected cytochrome *c* oxidase (EC 1.9.3.1) activity was that of lysine-13 at the top of the heme crevice. Trifluoroacetylation of lysine-13 increased the apparent Michaelis constant fivefold compared to that of native cytochrome *c*, but did not affect the maximum velocity. Trifluoroacetylation of lysine-55 at the left side of the cytochrome *c* molecule did not affect cytochrome oxidase activity in any way, nor did trifluoroacetylation of lysine-99 at the rear of the cytochrome *c* molecule. This indicates that the cytochrome oxidase binding site on cytochrome *c* involves only the front of the cytochrome *c* molecule and those lysines immediately surrounding the heme crevice.

The mechanism by which cytochrome *c* transports electrons from cytochrome *c* reductase to cytochome oxidase in mitochondria has remained elusive despite the wealth of chemical, biochemical, and x-ray data available on cytochrome *c*. The location of the reaction sites on cytochrome *c* for cytochrome *c* reductase and cytochrome oxidase is the subject of some controversy, particularly as to whether the sites are the same or different. A number of chemical modification and antibody binding studies indicate that the binding sites might be different (Takano et al., 1973, Margoliash et al., 1973; Wilson et al., 1975), while Salemme et al. (1973) have suggested that both oxidase and reductase bind on the front of cytochrome *c* at the heme crevice and that electrons are added and withdrawn directly from the heme.

Since the binding interaction of cytochrome *c* with cytochrome oxidase is known to involve the positively charged lysines on cytochrome *c*, one way to study the location of the binding sites is to measure how modification of specific lysine groups affects the reactivity of cytochrome *c* with the reductase and the oxidase. Specific trifluoroacetylation is an attractive method because it does not appear to cause any general protein conformational changes in cytochrome *c* and the resulting derivatives can be used for ^{19}F NMR[1] studies of the binding interactions of cytochrome *c*, as well as for enzyme-kinetic studies. A change in the oxidase or reductase activities due to

† From the Department of Chemistry, University of Arkansas, Fayetteville, Arkansas 72701. *Received August 3, 1976.* This work was supported by United States Public Health Service Grant GM-20488.

[1] Abbreviations used are: TFA, trifluoroacetyl; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; TNP, trinitrophenyl; TosPheCH$_2$Cl, 1-1-tosylamido-2-phenylethyl chloromethyl ketone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Mops, 4-morpholinepropanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; NMR, nuclear magnetic resonance.