The binding of $O_2$ to reduced cytochrome P-450 is the initial step in the activation of oxygen; subsequent addition of a second electron leads to substrate hydroxyla-
tion. Without the second electron, the complex be-
tween reduced cytochrome and $O_2$ (oxyferro) under-
goes an internal electron transfer to regenerate the oxid-
dized enzyme and, presumably, superoxide. We have
used 38% ethylene glycol and subzero temperatures to
stabilize the oxyferro complex of cytochrome P-450$_{sec}$
and have examined the effect of cholesterol, and hy-
droxycholesterol intermediates in the conversion of
cholesterol to pregnenolone, on the complex. The
binding of cholesterol or the intermediates 20a-hydroxycho-
lesterol, 22R-hydroxycholesterol, and 20a,22R-dihy-
droxycholesterol to the cytochrome perturbed the op-
tical spectra of the oxyferro complex with Soret max-
mums varying from 416 to 423 nm. Activation energies
for the autooxidation of each of these sterol-oxyferro
complexes were similar (approximately 22 kcal/mol).

The half-time for autooxidation of the oxyferro complex
was increased 15-fold by cholesterol over substrate-
free cytochrome, and the hydroxycholesterols caused a
further 3-17-fold increase in the stability of the oxy-
ferro complex over that observed for cholesterol, the
stability increasing with the number of hydroxyl
groups on the cholesterol side chain. This was observed in
both 38% ethylene glycol at $-17 \, ^\circ C$ and dioleoyl phos-
phatidylcholine vesicles at $2 \, ^\circ C$. The data indicate that
the 1-electron-reduced-oxygenated complex of cyto-
chrome P-450$_{sec}$ is kinetically stabilized by the binding of
the reaction intermediates, preserving the complex
for the arrival of the second electron.

Cytochrome P-450$_{sec}$, from the mitochondria of the adrenal
cortex catalyzes the three hydroxylations necessary for the
cleavage of the side chain of cholesterol to produce pregnen-
olone (1–3). The reaction occurs in the inner mitochondrial
membrane (4, 5), is rate limiting in the synthesis of the
gluccorticoids, and is regulated by adrenocorticotropin (6–8).
The first hydroxylation occurs in the 22R position, the second
in the 20a position, while the third results in cleavage of the
C20-C22 bond (2, 9, 10). Initial hydroxylation in the 20a
position rather than the 22R position may contribute a minor
pathway in the conversion of cholesterol to pregnenolone (9,
11). We have previously shown, using phosphatidylcholine
reconstituted cytochrome P-450$_{sec}$, that the hydroxycyto-
chrome intermediates bind 100–300 times tighter than chole-
sterol to the cytochrome (12). We have also shown, using these
intermediates, that below 37 $^\circ C$ the first hydroxylation in the
22 position is rate limiting while above 37 $^\circ C$ each hydroxy-
lation occurs at approximately the same rate (12).

Each hydroxylation catalyzed by cytochrome P-450$_{sec}$ re-
quires 2 electrons which are furnished by NADPH via adre-
nodoxin reductase and adrenodoxin and $O_2$ (10, 13, 14).
Adrenodoxin is a 1-electron carrier and appears to shuttle
between adrenodoxin reductase and the cytochrome (15).
Oxygen binds to the 1-electron-reduced form of cytochrome
P-450 (10, 14, 16). The complex is specific for the donor of the
second electron; in the adrenal, reduced adrenodoxin is re-
quired to complete the hydroxylation (10, 14). The binding of
$O_2$ to the 1-electron-reduced form of the cytochrome causes
perturbations in its optical spectrum; these have been exten-
sively studied in the soluble P-450$_{som}$ from Pseudomonas
putida (14, 17–19) and liver microsomal P-450$_{im}$ (19–23) as
although there are some reports for cytochrome P-450$_{im}$ (10,
16). The oxyferro complexes are unstable at room temperature
but can be stabilized for several hours at $-20 \, ^\circ C$ in ethylene
glycol or glycerol solutions (16, 18, 22).

In the present study, we have used low temperature to
stabilize the $O_2$–bound form of reduced cytochrome P-450$_{sec}$.

We have examined the effects of substrate and hydroxycho-
lesterol intermediates on the spectral characteristics of the
oxyferro complex and present spectra of the substrate and
intermediate-bound forms of the complex. Results on the
autooxidation rate of the oxyferro complex indicate that the
1-electron-reduced-oxygenated form of cytochrome P-450$_{sec}$ is
kinetically stabilized by complexion with the hydroxycho-
lesterols, while waiting for the second electron to complete
the hydroxylation.

**Experimental Procedures**

**Materials**—Cholesterol and 22-ketocholesterol were purchased
from Sigma Chemical Co. 20a,22R-Dihydroxycholesterol was the
generous gift of Dr. Enrico Forcieili of Syntex Research. Analytical
reagent grade ethylene glycol was from Mallinkrodt. The sources of
other materials used in this study have been previously reported (12).

**Purification of Cytochrome P-450$_{sec}$**—Cytochrome P-450$_{sec}$ was
purified from bovine adrenal cortex mitochondria by cholate extrac-
tion, ammonium sulfate fractionation, and hexyl agarose chromatog-
raphy as described previously (24). The purified cytochrome was
stored under liquid $N_2$ and retained essentially all its activity after
thawing. Using an extinction coefficient of 91 $\text{mM}^{-1} \text{cm}^{-1}$ for $A_{490}$
minus $A_{436}$ in the reduced-CO minus reduced difference spectrum (25,
26), the extinction coefficient of dithionite-reduced cytochrome P-
450$_{sec}$ at 411 nm was determined to be 163 ± 1 (mean ± S.D., $n = 4$)
$\text{mM}^{-1} \text{cm}^{-1}$. This value was used to calculate the concentration of the
cytochrome used in the formation of the oxyferro complex.

**Depletion of Substrate from the Cytochrome**—Cholesterol associ-
ated with the purified cytochrome was removed by incubation of the
cytochrome under conditions where catalytic conversion of the cho-
lesterol to pregnenolone could occur. A previously described proce-

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**Received for publication, March 22, 1982**
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dure (27) was modified as follows. The incubation of the purified cytochrome (36 μM), adrenodoxin (8 μM), adrenodoxin reductase (0.5 μM) [NAD<sup>+</sup>] (50 μM), oxoglutarate (2 mM), and glucose-6-phosphate dehydrogenase (2 units/ml) was carried out overnight at room temperature in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.0, 100 mM NaCl, 0.1 mM dithiothreitol, 0.1 mM EDTA. Glycerol was replaced by ethylene glycol (20% by volume) in the subsequent separation of the cytochrome from the other components by heparin column chromatography. The spectrum of the resulting cytochrome P-450<sub>ad</sub> was predominantly low spin with the Soret maximum at 416 nm. A shoulder at 492 nm (see “Results”) suggested that a small amount of cholesterol remained associated with the cytochrome. No cytochrome P-420 absorbance was apparent in the reduced-CO minus reduced difference spectra of the substrate-depleted cytochrome.

Preparation of Phospholipid Vesicles—Small unilamellar phospholipid vesicles were prepared in aqueous buffer from dioleoyl phosphatidylcholine and sterol by sonication in a bath-type sonicator as described previously (12, 26). Cytochrome P-450<sub>ad</sub>, incorporated into the vesicle membranes by incubation of the cytochrome (14 μM) with vesicles (1 mM phosphatidylcholine) for 30-60 min at room temperature, in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, 100 mM NaCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, conditions previously determined to give complete incorporation (28). Formation of Oxyferro Complex—The instability of the complex between reduced cytochrome and O<sub>2</sub> necessitated that most determinations be done below 0 °C. Ethylene glycol (38% by volume) was therefore added to the buffer (100 mM phosphate, pH 7.2, 0.1 mM dithiothreitol, 0.1 mM EDTA) to prevent freezing. The pH of such solutions has been reported to vary only slightly with temperature from the pH of the initial aqueous buffer (29). Hydroxysterols were added to the cytochrome in 10 μl of ethanol to give a final sterol concentration of 13 μM. Solutions of cytochrome P-450<sub>ad</sub>, (3-5 μM) in buffer (plus ethylene glycol as required) were made anaerobic by repeated evacuation and flushing with O<sub>2</sub>-free argon gas. The cytochrome was reduced at room temperature (22-25 °C) by adding sodium dithionite to a final concentration of 74 μM, and then cooled to the required temperature in the spectrophotometer cuvette holder. The temperature of the cuvette holder was controlled by circulation of alcohol from an American Instruments alcohol bath. The lowest stable temperature obtainable with this apparatus was -17 °C. The spectral characteristics of this species are similar to those reported for the oxyferro complex of cytochrome P-450<sub>ad</sub> (16), cytochrome P-450<sub>ad</sub> (17, 18), and cytochrome P-450<sub>ad</sub> (22) formed under similar conditions, indicating that this is indeed the species observed. Also, the addition of CO to the cytochrome-Fe<sup>2+</sup>-O<sub>2</sub> complex resulted in formation of the Fe<sup>2+</sup>-CO complex, as evidenced by a shift in the Soret absorption peak from 423 to 445 nm.

The oxyferro complex of the cholesterol-bound cytochrome decayed by a first order monophasic process to yield the initial high spin oxidized form of the cytochrome (Fig. 1), with a half-time of 38 min (Fig. 2). The autoxidation of the complex to yield the oxidized cytochrome (high spin) occurred with clear isosbestic points at 352, 410, 493, 534, and 590 nm (Fig. 1). Reduction of the cytochrome with dithionite after completion of the autoxidation showed that less than 4% of the original absorbance of the reduced cytochrome was lost during the process and no cytochrome P-420 absorbance was observed in the reduced CO minus reduced difference spectrum.

**RESULTS**

**Effect of Ethylene Glycol on the Spectra of Cytochrome P-450<sub>ad</sub>**—Purified cytochrome P-450<sub>ad</sub>, in aqueous solution is predominantly in the high spin form due to the binding of its substrate, cholesterol, which co-purifies with the cytochrome (11, 24, 30, 31). The addition of 38% ethylene glycol to the cytochrome at room temperature caused conversion of the cytochrome to the low spin form. This conversion was slowly reversed on cooling the cytochrome to generate the high spin (cholesterol-bound) form and was usually complete within 3 h at -17 °C. Subsequent warming of the sample to room temperature resulted in regeneration of the low spin form, indicating the transition is reversible.
The Oxyferro Complex of Substrate-depleted Cytochrome—Substrate associated with the purified cytochrome was removed by overnight metabolic depletion (see "Experimental Procedures"). The computer-corrected spectrum of the oxyferro complex of the substrate-depleted cytochrome, recorded at +17°C, had absorption maxima at 420 and 555 nm (Fig. 3). The formation and autooxidation of the Fe²⁺-O₂ complex was accompanied by a loss of 10-15% of the heme absorbance. The oxidation of Fe²⁺ to Fe³⁺ was biphase; the initial rapid phase had a half-time of 2.6 min and represented more than 80% of the reaction (Fig. 4). The half-time for the slow phase (35 min) is close to that observed for cholesterol-bound cytochrome (38-43 min) and may be due to incomplete removal of substrate. The spectrum of the oxidized cytochrome was not completely low spin as judged from the 392 nm absorbance (Fig. 3) which also suggests that some cholesterol may have remained associated with the cytochrome. Analysis of the autooxidation of the Fe²⁺-O₂ complex of cytochrome for which the metabolic depletion was carried out for 1 h rather than overnight supported the interpretation that the slow phase was due to cholesterol. The spectra designating the spin state of this cytochrome indicated it still had 50-60% of its endogenous cholesterol remaining, and the Fe²⁺-O₂ complex also decayed biphasically at +17°C (not shown). Here, the initial phase had a half-time of 3.0 min and represented 42% of the reaction while the slow phase had a half-time of 38 min.

The Effect of Binding of Intermediates on the Optical Spectra of the Oxyferro Complex—Since the cholesterol side chain cleavage reaction intermediates 20α-hydroxycholesterol, 22R-hydroxycholesterol, and 20α,22R-dihydroxycholesterol bind to cytochrome P-450, more tightly than does cholesterol (12), it was not necessary to remove the endogenous cholesterol from the purified cytochrome. At the concentration used (13 μM), the hydroxycholesterols completely displaced cholesterol from the active site of the cytochrome as judged from the complete conversion of the cytochrome from the high spin form (cholesterol-bound) to the low spin form by 20α-hydroxycholesterol and 22R-hydroxycholesterol, both low spin inducers (31). Like the oxidized enzyme, the Fe²⁺-O₂ complex formed from each of the hydroxycholesterol-bound forms of cytochrome P-450, had its own individual spectral characteristics (Fig. 5). The Soret absorbance maximum varied from 416 to 423 nm for the various forms of the oxyferro complex. Oxyferro complexes were formed by adding O₂ to sterol-bound reduced cytochrome P-450, (4.6 μM) at +17°C in 38% ethylene glycol. Spectra of the Fe²⁺-O₂ complex for each sterol were recorded at 2 nm/s in two identical experiments and the average is shown. Spectra of the oxyferro complexes for 20α-hydroxycholesterol and cholesterol (CHOL) were corrected for autooxidation to the time of O₂ addition as significant (2-6%) autooxidation occurred during the recording time. A, absolute spectra; B, oxyferro minus reduced difference spectra.

FIG. 4. The rate of autooxidation of the oxyferro complex of substrate-depleted cytochrome P-450, at +17°C. The rate of autooxidation of the Fe²⁺-O₂ complex formed as in Fig. 3 was measured from the absorbance change at 440 nm in repetitive scans of the cytochrome at 5 nm/s. Solid circles, entire reaction; open circles, fast phase corrected for the contribution of the slow phase.

FIG. 5. The effect of hydroxycholesterols on the spectra of the oxyferro complex. Oxyferro complexes were formed by adding O₂ to sterol-bound reduced cytochrome P-450, (4.6 μM) at +17°C in 38% ethylene glycol. Spectra of the Fe²⁺-O₂ complex for each sterol were recorded at 2 nm/s in two identical experiments and the average is shown. Spectra of the oxyferro complexes for 20α-hydroxycholesterol and cholesterol (CHOL) were corrected for autooxidation to the time of O₂ addition as significant (2-6%) autooxidation occurred during the recording time. A, absolute spectra; B, oxyferro minus reduced difference spectra.
The Oxyster Complex of Adrenal Cytochrome P-450

extension coefficients of oxyster complexes of cytochrome P-450

| Substrate                  | Oxyster minus | Soret max. | \\n|----------------------------|---------------|------------|
|                            | Reduced       | \(\epsilon^*\) | Max. \(\epsilon_{\text{max}}\) |
| Substrate depleted\(^d\)  | 420 nm        | 98 \pm 1*  | 433 nm |
| Cholesterol\(^d\)          | 423 nm        | 100 \pm 0  | 430 nm |
| 20a-Hydroxycholesterol\(^d\)| 420 nm        | 97 \pm 1   | 451 nm |
| 22R-Hydroxycholesterol\(^d\)| 416 nm        | 93 \pm 1   | 433 nm |
| 20,22-Dihydroxycholesterol | 420 nm        | 83 \pm 0   | 437 nm |

* Extinction coefficients were calculated using an extinction of 103 \(\text{mM}^{-1} \text{cm}^{-1}\) for the reduced cytochrome at 411 nm (see "Experimental Procedures").

20a,22R-dihydroxycholesterol was used as substrate for cytochrome P-450, to determine if any of the hydroxylation product (pregnenolone) was formed during autooxidation of the Fe\(^{3+}\) -O\(_2\) complex. The pregnenolone in triplicate extractions of the complex. The pregnenolone in triplicate extractions of the complex, which may have occurred before spectra were recorded. The spectral characteristics of the oxyster complexes did not appear to change with temperature over the range studied.

The Effect of Binding of Intermediates on the Stability of the Oxyster Complex—The hydroxycholesterol intermediates of the cholesterol side chain cleavage reaction markedly increased the stability of the Fe\(^{3+}\) -O\(_2\) complex (Fig. 6, Table II). The stability increased with the number of hydroxyl groups on the side chain of cholesterol. The activation energy for the autooxidation was relatively independent of the sterol bound to the cytochrome (Fig. 6) and values are slightly higher than reported for the autooxidation of the Fe\(^{3+}\) -O\(_2\) complex of P-450,\(_{\text{ox}}\) (18-19 kcal/mol) (18).

![Fig. 6. Arrhenius plot of the rate constants for autoxidation of the sterol-oxyster complexes of cytochrome P-450,\(_{\text{ox}}\). Autoxidation rates were measured in 38% ethylene glycol as described under "Experimental Procedures." CHOL, cholesterol.](image)

Table II

| Substrate                    | Half-time with 38% ethylene glycol | Half-time with dioleoyl phosphatidylcholine vesicles at 2 °C |
|------------------------------|----------------------------------|-------------------------------------------------------------|
|                              | \(-17^\circ\) C | 2 °C | min | min |
| No substrate                 | 2.6                        | 1.9 | 1.56 ± 0.2 |
| Cholesterol                  | 109                        | 5.8 | 4.2 |
| 20α-Hydroxycholesterol       | 168                        | 10.5| 5.4 |
| 22R-Hydroxycholesterol       | 583                        | 32.6| 24.4 ± 0.5 |

* The molar ratio of sterol to phosphatidylcholine was 0.4 for cholesterol and 0.05 for the other sterols.

† Values for the autoxidation of sterol-bound cytochrome in 38% ethylene glycol were taken from the Arrhenius plots (Fig. 6).

Mean ± deviation of duplicates.

Half-times for the decay of the Fe\(^{3+}\) -O\(_2\) complexes of cytochrome P-450,\(_{\text{ox}}\) at \(-17^\circ\) C and 2 °C are summarized in Table II. At 2 °C, it was possible to measure the half-times for the autoxidation of the Fe\(^{3+}\) -O\(_2\) complex for the cytochrome incorporated into fluid dioleoyl phosphatidylcholine vesicles, which more closely resemble the natural membrane environment of the cytochrome. In vesicles, increasing the number of hydroxyl groups on the side chain caused a comparable increase in the stability of the Fe\(^{3+}\) -O\(_2\) complex to that observed in 38% ethylene glycol. Half-times were, however, 20-50% lower than in 38% ethylene glycol. For aqueous, cholesterol-bound cytochrome at 2 °C, a half-time of 1.55 ± 0.19 min (mean ± deviation of duplicates) was observed which is also lower than in 38% ethylene glycol but similar to the value in vesicles and illustrates the stabilizing influence of the ethylene glycol on the oxyster complex.

Cardiolipin markedly promotes the binding of cholesterol to cytochrome P-450,\(_{\text{ox}}\), and appears to bind to the cytochrome itself (12, 32, 33). It does not, however, affect the stability of the Fe\(^{3+}\) -O\(_2\) complex. The half-time for autoxidation of the Fe\(^{3+}\) -O\(_2\) complex of cholesterol-bound cytochrome in dioleoyl phosphatidylcholine vesicles containing 30% (w/w) cardiolipin, at 2 °C was 1.6 min, the same as in the absence of cardiolipin (Table II).

Product Formation during Autoxidation—20a,22R-dihydroxycholesterol was used as substrate for cytochrome P-450,\(_{\text{ox}}\), to determine if any of the hydroxylation product (pregnenolone) was formed during autoxidation of the Fe\(^{3+}\) -O\(_2\) complex. The pregnenolone in triplicate extractions of the cytochrome with hexane was measured by a radioimmunoassay procedure previously described (24). The purified cytochrome contained 0.0059 ± 0.0003 (± S.D.) mol of pregnenolone/mol of cytochrome. After autoxidation of the Fe\(^{3+}\) -O\(_2\) complex, there were 0.025 ± 0.001 mol of pregnenolone/mol of cytochrome, indicating that with 20α,22R-dihydroxycholesterol as substrate a small (2%) but measurable amount of turnover accompanies the autoxidation of the Fe\(^{3+}\) -O\(_2\) complex.

**DISCUSSION**

The use of low temperatures and mixed solvent to stabilize the Fe\(^{3+}\) -O\(_2\) complex of cytochrome P-450,\(_{\text{ox}}\), has enabled us to study the interaction of substrate and intermediates with the complex. While we were limited to a minimum temperature of \(-17^\circ\) C, this was sufficient for analysis of the sterol-bound Fe\(^{3+}\) -O\(_2\) complexes. The Fe\(^{3+}\) -O\(_2\) complex of substrate-depleted cytochrome decayed significantly at \(-17^\circ\) C during the time required to record its spectrum but computerized analysis
The sterol-bound oxyferro complex of cytochrome P-450<sub>ox</sub> autooxidized to the oxidized enzyme by a first order monophasic reaction. The Fe<sup>2+</sup>-O<sub>2</sub> complex of substrate-depleted cytochrome autooxidized by a first order biphasic process, with the initial phase corresponding to substrate-free enzyme and the slow phase due to remaining cholesterol complex, left from incomplete removal of cholesterol from the cytochrome. This is in contrast to substrate-free cytochrome P-450<sub>ox</sub>, Fe<sup>2+</sup>-O<sub>2</sub> complex which autooxidizes in a biphasic manner in the absence of detectable substrate (22, 23), but resembles the monophasic decay of the Fe<sup>2+</sup>-O<sub>2</sub> complex of P-450<sub>ox</sub> (17, 18).

If all the cholesterol associated with purified cytochrome P-450<sub>ox</sub> were bound to the active site, then only one catalytic cycle would be required for its complete conversion to pregnenolone which should occur rapidly. However, the ratio of cholesterol to purified cytochrome can be expected to be greater than 1 (11, 31), indicating that some of the cholesterol must be bound by hydrophobic forces to secondary sites. Equilibration of cholesterol between the active and secondary sites, which might be expected to be slow based on the very low solubility of cholesterol, could therefore be rate limiting in the catalytic removal of cholesterol from the aqueous cytochrome. This would explain why overnight incubation of the cytochrome with the required electron transport components did not result in complete cholesterol removal.

The binding of cholesterol to cytochrome P-450<sub>ox</sub> caused a 15-fold increase in the stability of the Fe<sup>2+</sup>-O<sub>2</sub> complex at −17 °C. This is comparable to the 12-fold increase in half-time for the autooxidation of the Fe<sup>2+</sup>-O<sub>2</sub> complex of cytochrome P-450<sub>ox</sub> at −20 °C in 50% ethylene glycol, when camphor is bound (18). The binding of the hydroxycholesterol intermediates to cytochrome P-450<sub>ox</sub> caused a further 3-17-fold increase in the stability of the Fe<sup>2+</sup>-O<sub>2</sub> complex in the order 20a,22R-dihydroxycholesterol > 22R-hydroxycholesterol > 20a-hydroxycholesterol > cholesterol. This order does not relate to the spin state of the oxidized cytochrome-sterol complex, as cholesterol and 20a,22R-dihydroxycholesterol are high spin inducers while 22R-dihydroxycholesterol and 20α-hydroxycholesterol are low spin inducers (31). There is also a lack of correlation with the K<sub>d</sub> of the sterols which decrease in the order cholesterol > 20α-hydroxycholesterol > 20a,22R-dihydroxycholesterol > 22R-hydroxycholesterol (12). The stability of the Fe<sup>2+</sup>-O<sub>2</sub> complex does, however, correlate with the number of hydroxyl groups on the cholesterol side chain. For the monohydroxycholesterols, greater stability is given by the hydroxyl group in the 22R position (4-5-fold over cholesterol), which is the major intermediate in the conversion of cholesterol to pregnenolone (2, 10) than in the 20α position (3-fold) where hydroxylation appears to be a minor pathway (9, 11). The stabilization appears specific for the hydroxyl group as the Fe<sup>2+</sup>-O<sub>2</sub> complex of 22-ketocholesterol autooxidized at a rate similar to cholesterol (data not shown).

The Soret absorbance maximum of the oxyferro complex varied from 416 to 423 nm in the absolute spectrum and from 430 to 437 nm in the Fe<sup>2+</sup>-O<sub>2</sub> minus reduced difference spectrum depending on the sterol (Table I). It is interesting to note that the spectrum of the Fe<sup>2+</sup>-O<sub>2</sub> complex for 20α,22R-dihydroxycholesterol, which has a change transfer band at 650 nm and a clear shoulder at 580 nm, is similar to that of camphor-free or -bound P-450<sub>ox</sub> (17, 18). In contrast, the spectra for the other sterols, particularly cholesterol, more closely resemble the Fe<sup>2+</sup>-O<sub>2</sub> complex of P-450<sub>ox</sub> (22, 23).

Unlike cytochrome P-450<sub>ox</sub> and P-450<sub>cyt</sub>, cytochrome P-450<sub>ox</sub> catalyzes a triple hydroxylation with the product of one hydroxylation being the substrate for the next. We have previously shown that the hydroxycholesterol intermediates bind 100-300 times tighter to the oxidized cytochrome than does cholesterol (12, 33). This thermodynamic stabilization of the enzyme-sterol complexes thus prevents accumulation of free hydroxycholesterol intermediates during turnover. The present study shows that there is an increase in the stability of the Fe<sup>2+</sup>-O<sub>2</sub> complex in the presence of the hydroxycholesteroles over that observed for cholesterol. The 1-electron-reduced oxygenated cytochrome-sterol complexes are therefore kinetically stabilized, minimizing autooxidation, while awaiting the arrival of the second electron to complete the hydroxylation steps. The considerably tighter binding of hydroxycholesterol intermediates than cholesterol to the cytochrome and the increasing stability of the oxyferro-sterol complexes with successive intermediates lead to a highly directed reaction sequence, with little opportunity for "leakage." Once the initial binding of cholesterol occurs, a binding facilitated by complex formation of cytochrome P-450<sub>ox</sub> with adrenodoxin (27), complete hydroxylation becomes virtually inevitable.

Acknowledgment—We thank Christopher Batie for his help in the use of the computer for the recording and analysis of spectral data.

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