Takesue, S., and Omura, T. (1970), *J. Biochem. (Tokyo) 67*, 267.
Tzagoloff, A., MacLennan, D. H., McConnell, D. G., and Green, D. E. (1967), *J. Biol. Chem. 242*, 2051.
Wakil, S. J. (1964), *in* Metabolism and Physiological Significance of Lipids, Dawson, R. M. C., and Rhodes, D. N., Ed., Wiley, New York, N. Y., p 3.

Effects of Oxygenated Cholesterol Derivatives on Adrenal Cortex Mitochondria†

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ABSTRACT: The effects of a variety of cholesterol derivatives on the optical properties of adrenal cortex mitochondrial P-450 have been examined. Included in the sterols studied are several suggested oxygenated intermediates in the oxidative side-chain cleavage of cholesterol (forming pregnenolone). Typical type I difference spectra were produced when 25-hydroxycholesterol, 24(*R*)-hydroxycholesterol, or 20α,22(*R*)-dihydroxycholesterol was added to aerobic adrenal cortex mitochondria. Type II spectra were produced by 22-ketocholesterol, 22(*S*)-hydroxycholesterol, and 22(*R*)-hydroxycholesterol. Titration of the sterol-induced spectral change demonstrates saturation behavior and the lowest apparent dissociation constants were found for 22(*R*)-hydroxycholesterol (8 μM) and 20α,22(*R*)-dihydroxycholesterol (3 μM). Unlike substrates for steroid 11β-hydroxylation (or the other sterols examined) 22(*R*)-hydroxycholesterol and 20α-hydroxycholesterol produce a decrease in absorbance at 420 nm in the presence of electron donors and an increase in absorbance at 415–420 nm in the absence of reducing sources. Oxygen consumption by mitochondria showing respiratory control (with malate) was greatly accelerated by these two sterols and by 20α-hydroxycholesterol. This effect, very similar to that produced by 11-deoxycorticosterone, is transient and can be repeatedly demonstrated by adding more sterol. Dual-wavelength studies indicate that the spectral change produced by these three sterols is also transient in the presence of reducing equivalents, suggesting that each can bind to cytochrome P-450 and be subsequently oxidized by this tissue.

The side-chain cleavage of cholesterol (producing pregnenolone[1]) is thought to be the slow step in the synthesis of adrenal steroids and the site of regulation by ACTH (Stone and Hecter, 1955; Karaboyas and Koritz, 1965; Koritz and Kumar, 1970). While it has been postulated that side-chain cleavage involves the sequential hydroxylation of the 20 and 22 carbons followed by oxidative scission between these atoms (Solomon *et al.*, 1956; Shimizu *et al.*, 1960, 1961, 1962; Constantopoulos and Tchen, 1961; Chaundhuri *et al.*, 1962), it has not yet been possible to identify the true intermediates or to convincingly dissect the overall process into its component steps.

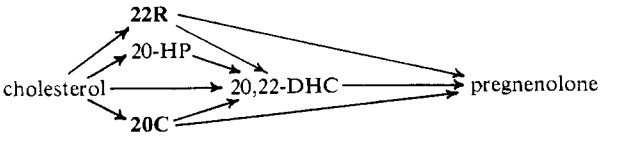
The difficulties arise from the rather slow rate of pregnenolone synthesis from cholesterol (Shimizu *et al.*, 1961; Kimura *et al.*, 1966), the inhibition by pregnenolone and 20α-hydroxycholesterol (**20C**) of their own synthesis (Koritz and Hall, 1964; Hall and Koritz, 1964; Ichii *et al.*, 1967; Raggatt and Whitehouse, 1966; Simpson and Boyd, 1967), and the general inability to isolate more than trace amounts of suspected intermediates after incubation of adrenal cortex with radioactive cholesterol (Hall and Koritz, 1964; Simpson and Boyd, 1967). Recently, Burstein *et al.* (1970a,b) have provided evidence that there are multiple routes from cholesterol to pregnenolone and that initial 20α-hydroxylation is probably a minor pathway. They suggest that 22(*R*)-hydroxylation is an important first step although evidence for a complex, concerted oxidation of the cholesterol side chain was also obtained.

It has been found that **20C**, 22(*R*)-hydroxycholesterol (**22R**), and cholesterol 20α-hydroperoxide (20-HP) are all effective precurors of pregnenolone in adrenal tissue (Shimizu *et al.*, 1961; Constantopoulos and Tchen, 1961; Chaudhuri *et al.*, 1962; Van Lier and Smith, 1970a; Burstein *et al.*, 1970a,b). There is some agreement that 20α,22(*R*)-dihydroxycholesterol (20,22-DHC) is an even better precursor of pregnenolone (Shimizu *et al.*, 1962; Constantopoulos *et al.*, 1962; Burstein *et al.*, 1970b), and, each of these sterols (except for the hydroperoxide) has been isolated from the adrenal cortex (Roberts *et al.*, 1969; Dixon *et al.*, 1970).

The suggested steps in the side-chain cleavage of cholesterol are summarized in Scheme I.

Cytochrome P-450 is known to be required somewhere in the overall process depicted above (Simpson and Boyd, 1967;

SCHEME I



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[1] The following trivial names and abbreviations are used: pregnenolone, 3β-hydroxypregn-5-en-20-one; **20C**, 20α-hydroxycholesterol; **22R**, 22(*R*)-hydroxycholesterol; **22S**, 22(*S*)-hydroxycholesterol; 20,22-DHC, 20α,22(*R*)-dihydroxycholesterol; **22K**, 22-ketocholesterol; 11-deoxycorticosterone (DOC), 21-hydroxypregn-4-ene-3,20-dione; 11-deoxycortisol, pregn-4-ene-3,20-dione-11α,17α,21-triol; Hepes, *N*-2-hydroxyethylpiperizine-*N'*-2-ethanesulfonic acid; 20-HP, cholesterol 20α-hydroperoxide.

Wilson and Harding, 1970a) and Ichii and coworkers (1967) have presented evidence that both the "20α-hydroxylase" and "22(R)-hydroxylase" require this cytochrome and adrenodoxin just as does steroid 11β-hydrolylation (Omura *et al.*, 1965; Kimura and Suzuki, 1967; Wilson and Harding, 1970b). Substrates of more completely characterized P-450-catalyzed reactions are thought to bind at or near the heme in this cytochrome and are known to alter its properties (Narasimhulu *et al.*, 1965; Schenkman *et al.*, 1967; Imai and Sato, 1967; Oldham *et al.*, 1968). One of the effects of P-450 substrates is the change produced in the optical absorption spectrum of this hemoprotein. Substrates may also change the rate at which P-450 accepts electrons (Gigon *et al.*, 1969; Strobel *et al.*, 1970; Ando and Horie, 1971) and, of course, there is an acceleration of oxygen consumption since molecular oxygen is used to oxidize the substrate (Oldham *et al.*, 1968; Cammer and Estabrook, 1967; Harding *et al.*, 1968).

It has been proposed that the spectrally evident substrate interaction is a necessary prerequisite of the subsequent mixed-function oxidation of that substrate (Schenkman *et al.*, 1967; Wilson *et al.*, 1969). The data supporting this view are most convincing with type I substrates generally. These substrates, in contrast to the substances producing type II difference spectra, also accelerate P-450 reduction and appear to produce a low-to-high spin transition in the heme iron (Whysner *et al.*, 1968, 1970; Mitani and Horie, 1969; Jefcoate and Gaylor, 1970). Interestingly, cholesterol produces no spectral change in adrenal mitochondria, presumably because endogenous concentrations of this sterol are sufficient to saturate the binding system unless the preparation is lipid depleted (Mitani and Horie, 1969; Young *et al.*, 1970). Type II spectra are regularly produced by **20C** and several steroids in adrenal mitochondria; this kind of spectral change is unusual for P-450 substrates in this tissue (Whysner *et al.*, 1970).

A report by Burstein *et al.* (1972) indicates that a variety of oxycholesterol derivatives produce either type I or type II difference spectra when added to preparations of adrenal mitochondrial P-450. Spectral evidence of the binding affinity of the sterols suggested to these investigators that **22R** is a more likely intermediate in pregnenolone synthesis than other hydroxycholesterols.

The present report describes the spectral effects of several oxycholesterol derivatives using bovine adrenal cortex mitochondria. Additionally, the ability of the sterols to stimulate oxygen utilization in suitably treated preparations has been examined. The results indicate that there are no apparent qualitative differences in the effects of **20C** and **22R** in this tissue. Both produce atypical spectral changes although **22R** may be a better substrate based on its greater stimulation of oxygen consumption and the greater reproducibility of its effects.

Methods and Materials

Bovine adrenals were obtained at a local meat packing plant and transported on ice to the laboratory within 30–45 min. After demedullation and scraping of the cortex from the capsule, a 10% (w/v) homogenate in 0.25 M sucrose–1 mM EDTA–1 mM Hepes at pH 7.4 (SHE medium) was prepared by use of a power-driven Teflon pestle followed by hand homogenization with a TenBroeck all-glass apparatus. Mitochondria were isolated by centrifugation at 9000g × 10 min of the supernatant of a 800g × 10 min spin. The mitochondrial pellet was washed two to three times by resuspending in 50% of the original volume of SHE and resedimenting at 10,000g × 10 min. The final pellet was resuspended at a protein concentration of 30–50 mg/ml. Throughout the procedure any visable sedimented hemoglobin was carefully excluded.

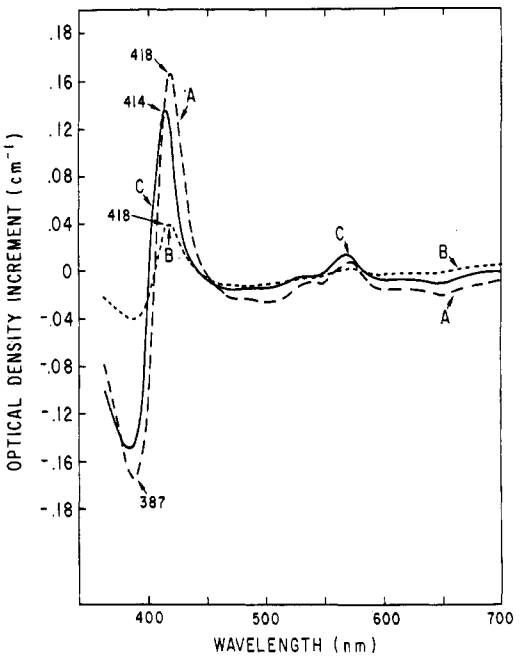

FIGURE 1: Difference spectra produced by 22(R)-hydroxycholesterol, 22(S)-hydroxycholesterol, and 22-ketocholesterol in adrenal cortex mitochondria. Tissue equivalent to 28.4 mg of P was suspended in 16 mM phosphate buffer (pH 7.4) containing $MgCl_2$ (6 mM), KCl (10 mM), sucrose (0.11 M), EDTA (0.4 mM), and Hepes (4.5 mM). The suspension was divided equally into two cuvets and a base line of equal light absorbance was obtained. Curve A was obtained by adding 22-ketocholesterol to the sample cuvet (final concentration of 108 μM) and an equal volume of ethanol to the reference. Curve B was obtained in a similar way using 22(S)-hydroxycholesterol (285 μM) and curve C was obtained with 22(R)-hydroxycholesterol (48 μM).

Difference spectra were obtained using a Cary 14 spectrophotometer equipped with the scatter-transmission accessory and the 0–0.1 slide-wire. Dual-wavelength spectra were obtained using the Phoenix spectrophotometer and oxygen uptake studies were performed on the Gilson oxygraph with the Clark electrode.

Most sterols were purchased from Ikapharm; 25-hydroxycholesterol was a gift from Dr. John Carrell (Upjohn) and 20α,22(R)-dihydroxycholesterol kindly provided by Dr. Enrico Forchielli (Syntex). Sterols were dissolved in ethanol and added in microliter volumes to the test samples with ethanol added to controls as appropriate.

Occasionally, frozen-thawed mitrochondria or submitochondrial particles (P_1 or P_3 of Omura *et al.*, 1966) were used for the spectral studies. Protein was determined by the method of Sutherland *et al.* (1949).

All difference spectra have been corrected for the base line and have been replotted (Harding and Nelson, 1966).

Results

Static difference spectra produced by several oxycholesterol derivatives are illustrated in Figure 1. Each of these is a type II spectrum similar to that produced by 20α-hydroxycholesterol (Whysner *et al.*, 1968). Spectrum A is that produced by 22-ketocholesterol and has absorbance maxima at 418, 530, and 570 nm and a minimum at 387 nm. This spectrum is

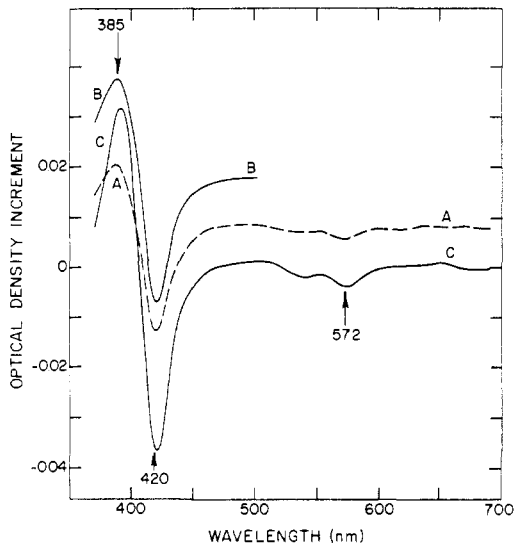

FIGURE 2: Difference spectra produced by 24(*R*)-hydroxycholesterol (**24R**), 25-hydroxycholesterol (**25C**), and 20α,22(*R*)-dihydroxycholesterol (20,22-DHC) in adrenal cortex mitochondria. Particles were suspended and spectra were obtained as described in Figure 1. Curve A, tissue concentration of 10.6 mg of P/cuvet and 12.5 μM 24(*R*)-hydroxycholesterol; curve B, 5.1 mg of P/cuvet and 21 μM 25-hydroxycholesterol; curve C, 10.6 mg of P/cuvet and 18.4 μM 20α,22(*R*)-dihydroxycholesterol.

quite similar to that ascribed to a change in cytochrome P-450 from high spin (Fe^{III}) to low spin (Whysner *et al.*, 1968; Mitani and Horie, 1969). The spectrum produced by 22S-hydroxycholesterol is depicted by line B. It is similar to that produced by **22K** although it is much reduced in magnitude. Curve C illustrates the difference spectrum produced by 22-(*R*)-hydroxycholesterol and, again, the similarity to curve A (**22K**) is obvious. Here, the bands are shifted significantly

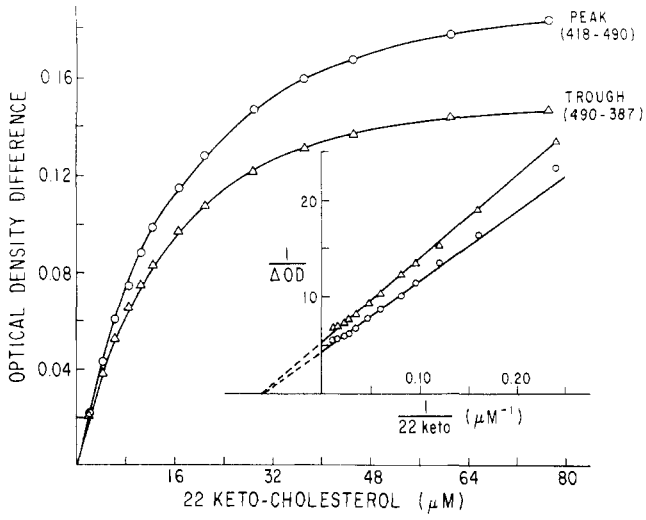

FIGURE 3: Titration of the spectral change produced by 22-ketocholesterol (**22K**). Mitochondria equivalent to 28.4 mg P were suspended as described in Figure 1 and divided equally into two cuvets. After obtaining a base line, successive additions of sterol (in ethanol) were made to the measure cuvet and equal volumes of ethanol were added to the reference sample. The spectrum was scanned following each addition. The changes in absorbance at 418 nm (peak) and 387 nm (trough) relative to the absorbance at 490 nm are plotted. The inset gives the same data in reciprocal form enabling estimation of an apparent spectral dissociation constant (K_s) of 18 μM.

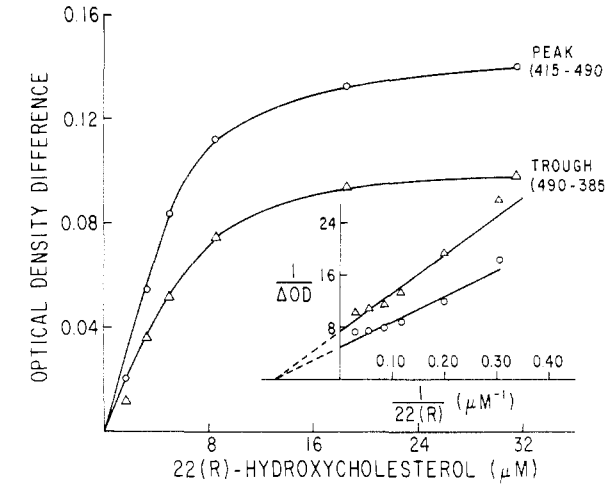

FIGURE 4: Titration of the spectral change produced by 22(*R*)-hydroxycholesterol. Tissue concentration and technic were exactly as described in Figure 3 although maximal changes following each addition required 5–15 min to develop. The double-reciprocal plot indicates an apparent K_s of 8 μM.

to the blue so that the maxima are at 414, 528, and 566 nm. The difference spectrum produced by **20C** (not shown) is very nearly identical to that produced by **22K** (Whysner *et al.*, 1968).

The static difference spectra produced by three other oxycholesterol derivatives are shown in Figure 2. Curves A and B were obtained employing 24(*R*)-hydroxycholesterol and 25-hydroxycholesterol and are seen to resemble the usual type I difference spectrum. Curve C illustrates the spectrum produced by 20α,22(*R*)-dihydroxycholesterol (20,22-DHC). This is also a typical type I difference spectrum with a maximum at 388 nm and absorbance minima at 420, 535, and 572 nm.

Since the spectral changes are stable in the absence of added electron sources, apparent spectral dissociation constants (K_s) can be obtained by titration. The titration with 22K is shown in Figure 3, in which the double-reciprocal plot is also given. Similar plots for **22R** and 20α,22(*R*)-dihydroxycholesterol are shown in Figures 4 and 5, respectively. The

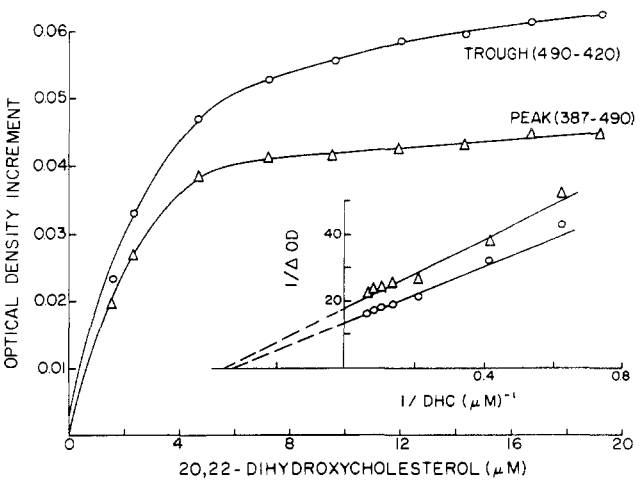

FIGURE 5: Titration of the spectral change produced by 20α,22(*R*)-dihydroxycholesterol. Conditions were as described in Figure 3 employing once frozen-thawed mitochondria at a concentration of 6.2 mg of P/cuvet. The inset indicates an apparent K_s of 3 μM.

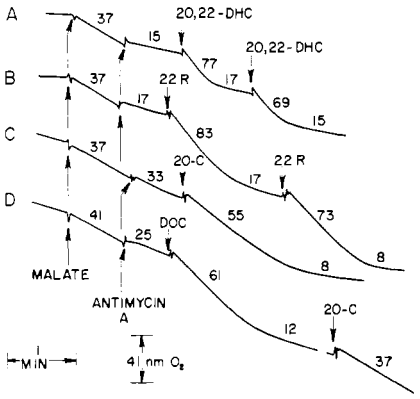

FIGURE 6: Comparison of sterol effects on oxygen consumption by adrenal cortex mitochondria. Mitochondria were suspended in the buffer mixture described in Figure 1 and oxygen was estimated polarographically with a Clark-type electrode. Additions were made in the order indicated; final concentrations of malate was 17 mM and antimycin A was 5 μg. Curves A and B utilized mitochondria containing 2.7 mg of P in each case and demonstrated a respiratory control ratio (with malate) of 3.2. Curves C and D were each obtained with mitochondria equivalent to 2.1 mg of P and having a respiratory control ratio (with malate) of 1.6. The numbers above parts of each tracing indicate the rate of oxygen consumption in nmoles of O_2/min. Each sterol was added as indicated in a final concentration of 33 μM.

TABLE I: Sterol-Stimulated Oxygen Utilization.

Sterol	[Sterol], μM	O_2 Uptake (Cor)[a]
DOC	20–33	26.7
22R	25–33	20.8
20,22-DHC	32	15.4
20C	25–33	13.6
24[b]	17	7.3
25[b]	33	6.8
22S	25–167	4.5
22K	25–167	0

[a] Rate of oxygen utilization in nmoles of O_2/min per nmole of P-450 after subtracting the presterol rate (supported by malate, 17 mM, and antimycin A, 5 μg). Respiratory control ratios of these mitochondria were 2–4 with succinate and 2–3 with malate. [b] 24- and 25-hydroxycholesterols.

spectral dissociation constants are estimated at 18 μM (**22K**), 8 μM (**22R**), and 3 μM (20,22-DHC). A similar titration with 22S indicates a very high apparent K_s of about 160 μM.

Since it has been shown that steroid substrates for 11β-hydroxylation stimulate an increased oxygen utilization (Oldham *et al.*, 1968; Cammer *et al.*, 1967; Harding *et al.*, 1968) —as would be anticipated from the requirement for molecular oxygen in this P-450-catalyzed mixed-function oxidase—the effect of the oxycholesterol derivatives on oxygen consumption was examined. The mitochondria typically had respiratory control ratios of 3–4 (succinate) or 2–3 (malate) and sometimes demonstrated a significant oxygen consumption (with malate) even in the presence of antimycin A, cyanide, or rotenone (*cf.* traces C and D in Figure 6). Sterol-stimulated oxygen utilization, in the presence of malate and antimycin A, is shown in Figure 6. The figure indicates that **20C** (lines C and D) stimulates oxygen consumption less than 20,22-DHC (line A) or **22R** (line B) both of which have comparable effects. The sterol-stimulated oxygen utilization occurs rapidly in each case and represents about two- to fivefold increase in the rate of respiration. The bursts of accelerated respiration are transient with these concentrations of sterol and are reinitiated by adding more sterol. These changes are similar in their magnitude and time course to the changes produced by the 11β-hydroxylation substrate, 11-deoxycorticosterone (line D).

It is of interest that **22K** does not stimulate oxygen consumption in this sytem even though it does produce a difference spectrum similar to that induced by **20C** or **22R**. Oxygen uptake is slightly augmented by **22S**, which produces only a small spectral change even in high concentrations. Most significant is the marked stimulation of oxygen utilization by the suspected intermediates in cholesterol side-chain cleavage; *i.e.*, **22R**, **20C**, and 20,22-DHC. A summary of the effects of the various sterols on oxygen utilization is given in Table I.

In the presence of a suitable source of electrons, the optical changes produced by those sterols accelerating oxygen consumption may be quite different from the static spectra described earlier. Figure 7 shows several tracings of the difference with time in the absorbance of adrenal cortex mitochondria at two wavelengths. Trace A illustrates, for purposes of comparison, the cyclic absorbance changes occurring after the addition of 11-deoxycortisol. The effects of antimycin A and then succinate indicate the progressive reduction of cytochrome *b* (reduced Soret band at 430 nm) shown by upward deflections of the trace using the wavelength pair noted. Subsequent addition of malate—permitting reduction of the early portion of the respiratory chain as well as the hydroxylation (P-450) pathway—produces a further upward deflection indicating the appearance of a 420-nm peak (Oldham *et al.*, 1968). Addition of 11-deoxycortisol causes a rapid and transient downward deflection indicating

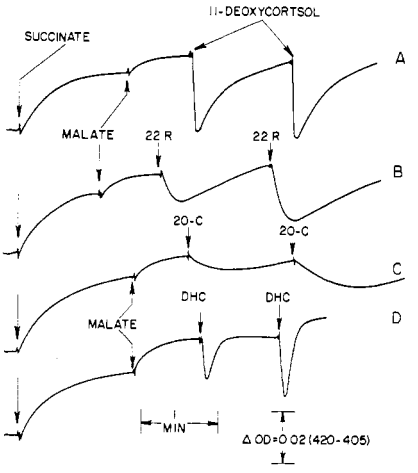

FIGURE 7: Comparison of transient sterol effects on spectral properties of adrenal mitochondria. Mitochondria equivalent to 6.2 mg of P were diluted to 3 ml in the buffer medium described in Figure 1 (containing 3 μg of antimycin A) and the difference in absorbancy between 420 and 405 nm was monitored with the dual-wavelength spectrophotometer. An increase in this difference is indicated by an upward deflection. Additions were made as indicated resulting in final concentrations of 8 mM (succinate), 8 mM (malate), 3.3 and 5 μM (11-deoxycortisol, curve A), 8.4 and 16.8 μM (22(*R*)-hydroxycholesterol, curve B), 4.2 and 12.6 μM (20α-hydroxycholesterol, curve C), 4 and 8 μM (20α,22(*R*)-dihydroxycholesterol, curve D).

loss of absorbance at 420 nm as the expected type I spectrum appears and then disappears. Previous studies (Oldham *et al.*, 1968; Harding *et al.*, 1968; Wilson *et al.*, 1969) have shown that the disappearance is the result of the conversion of this substrate to its 11β-hydroxylated product and this view is supported by the reinitiation of the spectral change after adding 11-deoxycortisol again.

Subsequent tracings illustrate the sequence of changes obtained by adding the oxycholesterol derivatives. Trace B was tained using **22R** and shows transient absorbancy changes quite similar to those produced by 11-deoxycortisol. Again the sterol-induced absorbance changes are temporary, capable of reinitiation by another addition of sterol. Of special interest here is the demonstration that the absorbance change is in the same direction as that produced by substrates for 11β-hydroxylation. That is, in this case **22R** has produced a loss in absorbance at 420 nm *not* the type II difference spectrum illustrated clearly in Figure 1 (curve B). A similar discrepancy is noted between the spectral change produced by **20C** in the presence or absence of reducing equivalents and is illustrated by curve C of Figure 7. Unlike the type II difference spectrum known to be produced by this sterol (Whysner *et al.*, 1968; Mitani and Horie, 1969), **20C** produces a temporary loss of absorbance at 420 nm in the presence of electrons derived from malate. This loss can be demonstrated repeatedly by giving additional supplies of the sterol. Curve D was obtained using 20α,22(*R*)-dihydroxycholesterol. The absorbance changes occurring with the additions of succinate and malate are again the same and the subsequent addition of sterol produces a very rapid and transient downward deflection. This change indicates a loss in absorbance at 420 nm as suggested by its type I difference spectrum (curve C, Figure 3). Like the results with 11-deoxycortisol, **20C**, and **22R**, the absorbance change is transient and can be recycled by adding more 20,22-DHC.

Discussion

The reasons for the variety of optical changes produced by the several oxycholesterol derivatives examined are not clear. Discounting, for the moment, the relative affinities of these derivatives for the P-450-containing tissue it is evident that 20α-22(R)-dihydroxycholesterol, 24-, and 25-hydroxycholesterol each produce a type I difference spectrum while 22-ketocholesterol, **20C**, **22R**, and 22(*S*)-hydroxycholesterol each produce a type II difference spectrum. By analogy with other known substrates of adrenal P-450-catalyzed reactions (Cooper *et al.*, 1965; Oldham *et al.*, 1968; Mitani and Horie, 1969), one might anticipate production of type I spectra by early intermediates in the conversion of cholesterol to pregnenolone provided that utilization of the intermediate requires cytochrome P-450. Some evidence has been provided indicating that this cytochrome is involved in the overall process (Simpson and Boyd, 1967; Wilson and Harding, 1970a) as well as in 20α-hydroxylation and 22(*R*)-hydroxylation (Ichii *et al.*, 1967).

Of the suggested intermediates examined here, only 20a, 22(*R*)-dihydroxycholesterol produces the usual type I difference spectrum. It is not known that P-450 is required in pregnenolone formation from this sterol. In fact, a mixed-function oxidative process might not be expected in the side-chain cleavage of this sterol if the true products are pregnenolone and isocaproic aldehyde as reported (Constantopoulos *et al.*, 1966). However, the question is not settled since there is evidence that, like other reactions known to be P-450-dependent in this tissue, this reaction requires TPNH and molecular oxygen (Constantopoulos *et al.*, 1962; Shimizu, 1968). The marked, but transient, stimulation of oxygen uptake caused by this sterol in mitochondria under conditions suitable for mixed-function oxidation may well be a direct reflection of the conversion of 20,22-DHC to pregnenolone. This interpretation is supported by the transient absorbance changes produced by small quantities of this sterol in similar conditions and by the ability to repeatedly produce both effects by additions of more sterol.

The difference spectrum produced by 20,22-DHC and its affinity are similar to results obtained by Burstein *et al.* (1972) using various derivatives of adrenal mitochondria but are in disagreement with the report (Van Lier and Smith, 1970b) that this sterol has no effect on the spectra of extracted acetone powders of adrenal cortex mitochondria. Also, the rather marked variability in the spectral effects of both **22R** and 20,22-DHC observed by Burstein and coworkers (1972) was not apparent in our studies with these sterols. The discrepant results may well be related to the use of very different tissue preparations by the several investigators (acetone powders, intact mitochondria).

The type I difference spectra produced by 24- and 25-hydroxycholesterol have been of variable magnitude and frequently diminish slowly even in the absence of added reducing equivalents. The spectrum produced by 25-hydroxycholesterol is in agreement with the report by Burstein *et al.* (1972). Since both of these sterols have been reported to be effective inhibitors of cholesterol, side-chain cleavage in this tissue (Raggatt and Whitehouse, 1966; Simpson and Boyd, 1967) their stimulation of oxygen consumption by these mitochondria was not expected. However, Burstein and Gut (1971) have shown that 25-hydroxycholesterol can be readily converted to pregnenolone by adrenal tissue. It seems reasonable to assume that the inhibition reported for these sterols is that expected of a cosubstrate for the side-chain-cleaving system, thus accounting for the type I spectral change and the accelerated oxygen utilization observed with each sterol.

The spectral data obtained here confirm and extend previous reports on 20α-hydroxycholesterol and the 22(*R*)-hydroxy derivative of cholesterol (Whysner *et al.*, 1968; Burstein *et al.*, 1972). Both produce type II difference spectra in the absence of reducing equivalents; both considerably stimulate oxygen uptake and their spectral dissociation constants are similar. Since both are known to be effectively converted to pregnenolone by this tissue (Shimizu *et al.*, 1961; Constantopoulos and Tchen, 1961; Burstein *et al.*, 1970a,b), the similarities found here provide little assistance in identifying which is the usual intermediate in the side-chain cleavage of cholesterol. The kinetic data suggesting that more cholesterol passes through 22(*R*)-hydroxycholesterol rather than through 20α-hydroxycholesterol (Burstein *et al.*, 1970b) is mildly supported by the somewhat more rapid oxygen utilization observed with **22R** together with the considerably more rapid spectral changes in the dual-wavelength studies (probably reflecting conversion of sterol) and the greater reproducibility of the effects of this sterol.

Although both 22(*R*)- and 20α-hydroxycholesterol produce type II difference spectra in the absence of electron sources, each produces a loss of absorbance at about 420 nm when added to adrenal mitochondria in the presence of electrons (malate). Each produces a sequence of events qualitatively similar to that produced by DOC and which have been clearly linked to the steroid-induced increase in oxygen utilization and conversion of this steroid to its 11β-hydroxylated product

(Cammer and Estabrook, 1967; Harding *et al.*, 1968). Similar studies relating the cyclic loss of absorbance produced by 20α-hydroxycholesterol to the accelerated oxygen uptake and the side-chain cleavage of this sterol (L. D. Wilson and B. W. Harding, in preparation) support the view that the loss in 420 nm absorbance—associated with enhanced oxygen utilization—is the spectral concomitant of the mixed function oxidation of both **20C** and 22(*R*)-hydroxycholesterol and probably, 20α,22(*R*)-duhydroxycholesterol as well.

Combining the data on static difference spectra, spectral dissociation constants, sterol-stimulated oxygen utilization, and transient absorbance changes under conditions allowing mixed-function oxidation provides indirect support for the view that 20α-hydroxycholesterol, 22(*R*)-hydroxycholesterol, and 20α,22(*R*)-dihydroxycholesterol can each serve as substrates for adrenal mitochondrial oxidation, probably P-450 dependent. Similar data on 22(*S*)-hydroxycholesterol and 22-ketocholesterol suggests that these are not substrates for the oxidative process although each can interact to some degree with cytochrome P-450.

Acknowledgment

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References

Ando, N., and Horie, S. (1971), *J. Biochem. (Tokyo) 70*, 557.
Burstein, S., Co, N., Gut, M., Schleyer, H., Cooper, D. Y., and Rosenthal, O. (1972), *Biochemistry 11*, 573.
Burstein, S., and Gut, M. (1971), *Recent Progr. Hormone Res. 27*, 303.
Burstein, S., Kimball, H. L., and Gut, M. (1970b), *Steroids 15*, 809.
Burstein, S., Zamoscianyk, H., Chaudhuri, N. K., and Gut, M. (1970a), *Steroids 15*, 13.
Cammer, W., and Estabrook, R. W. (1967), *Arch. Biochem. Biophys. 122*, 721.
Chaudhuri, A. C., Harada, Y., Shimizu, K., Gut, M., and Dorfman, R. I. (1962), *J. Biol. Chem. 237*, 703.
Constantopoulos, G., Carpenter, A., Satoh, P., and Tchen, T. T. (1966), *Biochemistry 5*, 1650.
Constantopoulos, G., Satoh, P. S., and Tchen, T. T. (1962), *Biochem. Biophys. Res. Commun. 8*, 50.
Constantopoulos, G., and Tchen, T. T. (1961), *J. Biol. Chem. 236*, 65.
Cooper, D. Y., Narasimhulu, S., Slade, A., Raich, W., Foroff, O., and Rosenthal, O. (1965), *Life Sci. 4*, 2109.
Dixon, R., Furutachi, T., and Lieberman, S. (1970), *Biochem. Biophys. Res. Commun. 40*, 161.
Gigon, P. L., Gram, T. E., and Gillette, J. R. (1969), *Mol. Pharmacol. 5*, 109.
Hall, P. F., and Koritz, S. B. (1964), *Biochim. Biophys. Acta 93*, 441.
Harding, B. W., Ball, J. J., Oldham, S. B., and Wilson, L. D. (1968), *in* Functions of the Adrenal Cortex, McKerns, K. W., Ed., New York, N. Y., Appleton-Century-Crofts.
Harding, B. W., and Nelson, D. (1966), *J. Biol. Chem. 241*, 2212.
Ichii, S., Omata, S., and Kobayashi, S. (1967), *Biochim. Biophys. Acta 139*, 308.
Imai, Y., and Sato, R. (1967), *J. Biochem. (Tokyo) 61*, 26.
Jefcoate, C. R. E., and Gaylor, J. L. (1970), *Biochemistry 9*, 3816.
Karaboyas, G. C., and Koritz, S. B. (1965), *Biochemistry 4*, 462.
Kimura, T., Satoh, P. S., and Tchen, T. T. (1966), *Anal. Biochem. 16*, 355.
Kimura, T., and Suzuki, K. (1967), *J. Biol. Chem. 242*, 485.
Koritz, S. B., and Hall, P. F. (1964), *Biochemistry 3*, 1298.
Koritz, S. B., and Kumar, A. M. (1970), *J. Biol. Chem. 245*, 152.
Mitani, F., and Horie, S. (1969), *J. Biochem. (Tokyo) 65*, 269.
Narasimhulu, S., Cooper, D. Y., and Rosenthal, O. (1965), *Life Sci. 4*, 2101.
Oldham, S. B., Wilson, L. D., Landgraf, W. L., and Harding, B. W., (1968), *Arch. Biochem. Biophys. 123*, 484.
Omura, T., Sanders, E., Estabrook, R. W., Cooper, D. Y., and Rosenthal, O. (1966), *Arch. Biochem. Biophys. 117*, 660.
Omura, T., Sato, R., Cooper, D. Y., Rosenthal, O., and Estabrook, R. W. (1965), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 24*, 1181.
Raggatt, P. R., and Whitehouse, M. W. (1966), *Biochem. J. 101*, 819.
Roberts, K. D., Bandy, L., and Lieberman, S. (1969), *Biochemistry 8*, 1259.
Schenkman, J. B., Remmer, H., and Estabrook, R. W. (1967), *Mol. Pharmacol. 3*, 113.
Shimizu, K. (1968), *Arch. Biochem. Biophys. 124*, 1016.
Shimizu, K., Dorfman, R. I., and Gut, M. (1960), *J. Biol. Chem. 235*, 25.
Shimizu, K., Gut, M., and Dorfman, R. I. (1962), *J. Biol. Chem. 237*, 699.
Shimizu, K., Hayano, M., Gut, M., and Dorfman, R. I. (1961), *J. Biol. Chem. 236*, 695.
Simpson, E. R., and Boyd, G. S. (1967), *Eur. J. Biochem. 2*, 275.
Solomon, S., Levitan, P., and Lieberman, S. (1956), *Rev. Can. Biol. 15*, 282.
Stone, D., and Hecter, O. (1955), *Arch. Biochem. Biophys. 54*, 121.
Strobel, H. W., Lu, A. Y. H., Heidema, J., and Coon, M. J. (1970), *J. Biol. Chem. 245*, 4851.
Sutherland, E. W., Cori, C. F., Haynes, R., and Olsen, N. S. (1949), *J. Biol. Chem. 180*, 825.
Van Lier, J. E., and Smith, L. L. (1970a), *Biochim. Biophys. Acta 210*, 153.
Van Lier, J. E., and Smith, L. L. (1970b), *Biochim. Biophys. Acta 218*, 320.
Whysner, J. A., Ramseyer, J., and Harding, B. W. (1970), *J. Biol. Chem. 245*, 5441.
Whysner, J. A., Ramseyer, J., Kazmi, G., and Harding, B. W. (1968), *Biochem. Biophys. Res. Commun. 32*, 921.
Wilson, L. D., and Harding, B. W. (1970a), *Biochemistry 9*, 1621.
Wilson, L. D., and Harding, B. W. (1970b), *Biochemistry 9*, 1615.
Wilson, L. D., Oldham, S. B., and Harding, B. W. (1969), *Biochemistry 8*, 2975.
Young, D. G., Holroyd, J. D., and Hall, P. F. (1970), *Biochem. Biophys. Res. Commun. 38*, 184.