Substrate-Induced Difference Spectra and Cholesterol to Pregnenolone Conversion with Adrenal Heme Protein P-450*

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ABSTRACT: A correlative study was made between the enzymatic rates of conversion of cholesterol to pregnenolone and the substrate-induced difference spectra (in the 390- to 420-nm range) observed with cholesterol-oxygenated derivatives using various heme protein P-450 preparations from bovine adrenocortical mitochondria. With acetone-dried powder extracts at 24 μM (22*R*)-20α,22-dihydroxycholesterol, 20α-hydroxy-22-oxocholesterol, 20β-hydroxy-20-isocholesterol, and 25-hydroxycholesterol exhibited type I difference spectra. Type II spectra were observed with 20α-hydroxycholesterol, (22*R*)-22-hydroxycholesterol, (22*S*)-22-hydroxycholesterol, 20α,21-dihydroxycholesterol, (22*S*)-20α,22-dihydroxycholesterol, and 22-oxocholesterol. With some acetone-dried powder preparations, no difference spectra were observed with (22*R*)-20α,22-dihydroxycholesterol. The type of the difference spectra observed with some of the sterols depended on the technique used in the preparation of the heme protein P-450 fractions. There appeared to be no consistent correlation between the ability of the preparations to catalyze the conversions of cholesterol to pregnenolone and the magnitude of the observed difference spectra. Although with the acetone-dried powder preparations the apparent binding affinity of the (22*R*)-22-hydroxycholesterol and that of the (22*R*)-20α,22-dihydroxycholesterol (as determined from the difference spectra) was higher than that exhibited by 20α-hydroxycholesterol, the converse was seen with some of the other partially purified heme protein P-450 preparations studied. These results suggest that great caution must be exercised in deriving mechanistic enzymatic conclusions from substrate-induced difference spectra as these may drastically vary both with respect to magnitude and affinity without significantly affecting, in certain cases, the enzymatic activities.

In recent studies with bovine adrenocortical acetone-dried powders (Burstein *et al.*, 1970a,b), the kinetics of the enzymatic transformations of cholesterol (20α-hydroxycholesterol,[1] (22*R*)-22-hydroxycholesterol, and (22*R*)-20α,22-dihydroxycholesterol) to pregnenolone have been investigated in an attempt to ascertain to what extent these hydroxylated derivatives participate as intermediates in the conversion of cholesterol to pregnenolone. The results of these studies have indicated that while only a relatively small fraction of pregnenolone arose *via* the consecutive reaction schemes (A) cholesterol → **20α** → **20,22R** → pregnenolone and (B) cholesterol → **22R** → **20,22R** → pregnenolone, pathway B appeared to proceed at a considerably higher rate than A. The major portion of the pregnenolone, accountable by these hydroxylated cholesterol derivatives participating as intermediates, appeared to arise by what could be best described as a one-step (or "direct") enzymatic conversion of cholesterol to the dihydroxy derivative followed by an oxidative cleavage of the latter to pregnenolone. In addition to this one-step transformation, other such direct enzymatic reactions appeared to exist, such as the direct conversion of (22*R*)-22-hydroxycholesterol to pregnenolone. One explanation of these one-step transformations was the postulation of the existence in the enzymatic preparations used of multienzyme complexes as previously suggested by Hall and Koritz (1964) (also *cf.* Ginsburg and Stadtman, 1970). Another possibility was the participation of free radicals as the reactive species (*cf.* Hochberg *et al.*, 1971; Lippman and Lieberman, 1970).

In studies to further elucidate these questions, investigations have been initiated toward the goal of the separation of the various enzymatic activities that may be involved. As an aid in these separations it was contemplated to utilize the absorption difference spectra in the 390- to 420-nm (Soret) region known to be induced by some of these sterols (*cf.* Whysner *et al.*, 1970; Jefcoate *et al.*, 1970; Van Lier and Smith, 1970).

This report is concerned with the relationship between the difference spectra of various mitochondrial heme protein P-450 preparations elicited by various oxygenated cholesterol derivatives and the enzymatic conversion of cholesterol to pregnenolone.

Experimental Section

Heme Protein P-450 Preparations. Preparations 1 and 2 were acetone-dried powders of bovine adrenocortical mitochondria. Preparation 1 was prepared by precipitation of the mitochondrial suspension (in distilled water) in 20 volumes of acetone precooled to −20°, filtration and washing with −20° acetone and ether followed by drying in a desiccator under vacuum at 5°. Preparation 2 was prepared by first lyophilizing the mitochondria followed by homogenization of the powder with acetone and ether precooled to −30°, filtration, and desicca-

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[1] The following trivial names and abbreviations were used: pregnenolone, 3β-hydroxypregn-5-en-20-one; **20α**, 20α-hydroxycholesterol; **22R**, (22*R*)-22-hydroxycholesterol; **22S**, (22*S*)-22-hydroxycholesterol; **20β**, 20β-hydroxy-20-isocholesterol; **20,22R**, (22*R*)-20α,22-dihydroxycholesterol; **20,22S**, (22*S*)-20α,22-dihydroxycholesterol; **20,22-oxo**, 20α-hydroxy-22-oxocholesterol; **22-oxo**, 22-oxocholesterol; **20,21**, 20α,21-dihydroxycholesterol; **25**, 25-hydroxycholesterol.

tion. Preparations 3–5 were prepared from bovine adrenocortical mitochondria according to the method of Jefcoate *et al.* (1970) and represented the 15–25, 25–35, and 35–60% ammonium sulfate fractions, respectively, described by these investigators. Preparations 6–11 were Triton N-101 extracts of acetone- and butanol-treated, sonicated, and lyophilized bovine adrenocortical mitochondria prepared according to the method of Schleyer *et al.* (1971).[2] Preparations 6–8 were 0.02% Triton N-101 extracts precipitated with 15–25, 25–35, and 0–35% ammonium sulfate, respectively. Preparations 9 and 10 were each 0.2% Triton N-101 extracts prepared on two different occasions, while preparation 11 was a 1% Triton N-101 extract.

The acetone-dried powders 1 and 2 were homogenized in 0.02 M phosphate buffer and centrifuged at 50,000*g* for 45 min to 1 hr. The clear supernatants (containing 4–15 mg/ml of protein) were used for the determination of the enzymatic activity and the substrate-induced spectra.

All preparations were stored at −20°. Since the effect of storage at this temperature was not thoroughly investigated, all experiments for which comparisons between enzymatic rate and substrate-induced spectra were reported were done on the same day.

Enzymatic Assays. The enzymatic conversion of cholesterol to pregnenolone was studied using [1,2-^{3}H]cholesterol at 23° in beakers in an air atmosphere using a Dubnoff metabolic shaker. (It has been shown by Simpson and Boyd (1967) that at this lower temperature the formation of pregnenolone from cholesterol is a linear function of time and does not exhibit a lag seen at 37°.) The cholesterol was dissolved with the aid of Tween 80 in 0.02 M phosphate buffer to which TPN, glucose 6-phosphate, and yeast glucose-6-phosphate dehydrogenase were added as previously described (Burstein *et al.*, 1968, 1970a). The heme protein P-450 preparations were added last, after a 2- to 3-min preincubation period was allowed for the full generation of TPNH. The requirement of nonheme iron and flavoprotein for the cholesterol side-chain cleavage of purified cytochrome P-450 preparations have been described by Bryson and Sweat (1968). Similar requirements for the cleavage of cholesterol sulfate to pregnenolone also have been described by Young and Hall (1969). However, the optimal molar requirements have not been precisely defined by these investigators. The heme protein P-450 preparations reported here were all incubated in the presence of 1 nmole/ml of purified nonheme iron protein and 0.043 nmole/ml of purified flavoprotein. This, in most cases, provided an approximate molar ratio in the incubation mixture of heme protein P-450 to nonheme iron protein to flavoprotein of 1:20:1. This was the optimal ratio described by Cooper *et al.* (1968) for the 11β hydroxylation of 11-deoxycorticosterone. With cholesterol, near-maximal rates were observed at a molar ratio of heme protein P-450 to nonheme iron protein of 10 and the activity did not materially increase when this ratio was further increased to 20 or 30. The concentration of flavoprotein did not appear to be critical.

The pregnenolone formation from cholesterol was studied at substrate concentrations leading to maximal velocities and was a reasonably linear function of time, allowing the determination of initial rates either from progress curves or from single points and zero-time blanks. At the heme protein P-450 concentrations used there existed a fairly good linear relationship between the enzymatic pregnenolone formation and the heme protein P-450 concentration. The exogenous substrate concentration in the incubation media was either obtained by using sufficient amounts of the undiluted radioactive cholesterol or by adding unlabeled substrate to achieve the desired concentration. The endogenous cholesterol content in the various preparations was determined by gas chromatography–mass spectrometry and was brought into account in expressing the rate of the pregnenolone formation. The concentration given in Table III was the total substrate concentration present in the incubation. The enzymatic pregnenolone formation was expressed as nmoles min^{-1} (heme protein P-450)$^{-1}$.

The formation of pregnenolone was determined using column partition chromatography on Celite 545 and thin-layer chromatography which, as previously described (Burstein *et al.*, 1968, 1970a), led to a radiochemically pure product as ascertained by reverse isotope dilution techniques and crystallizations to constant specific activity.

Substrates. The [1,2-^{3}H$_2$]cholesterol used in the incubations was the same as previously described (Burstein *et al.*, 1970a), had a specific activity of 12.6 Ci/mmole, and was again purified by partition and thin-layer chromatography prior to use. The unlabeled sterols used had the correct melting points, infrared (ir) and nuclear magnetic resonance (nmr) spectra.

Determination of Substrate-Induced Difference Spectra. The difference spectra were determined in a Cary Model 15 double-beam spectrophotometer using cuvets of 1-cm path length and 1.2-ml capacity. After obtaining a base line with the heme protein P-450 preparation in both reference and sample cuvets, the sterols were added in 5 μl of absolute ethanol to the sample cuvet while 5 μl of absolute ethanol was added to the reference cuvet. All induced difference spectra were determined at a substrate concentration of 24–26 μM which elicited maximal absorbance differences at this concentration level, *i.e.*, doubling or tripling this concentration did not lead to a further increase in absorbance. The absorbances reported were for differences between the maximum to minimum absorbances observed, which fell in the 420- to 390-nm vicinity (or *vice versa*), respectively. The actual wavelengths of the maxima or minima were not precisely at 420 or 390 nm. Thus, the 20α-hydroxycholesterol and the (22*R*)-22-hydroxycholesterol exhibited induced spectra with λ_{max} at 418 and 414 and λ_{min} at 387 and 385 nm, respectively, while the (22*R*)-20α,22-dihydroxycholesterol exhibited λ_{max} at 392 and λ_{min} at 420 nm. The absorption wavelength of the maxima and minima appeared to vary slightly between preparations. These variations, however, were not reported as no regularity was apparent. The rate at which the difference spectra developed varied with the particular sterol studied and also with the preparation, but was not accurately measured. The absorbances reported were for the maximally developed spectra, determined by repetitive scans, which as a rule took (with the "slowest" substrates) 15–20 min. The protein concentration used for the determination of the absorbances was with the acetone dried powders 3–15 mg/ml. With the other heme protein P-450 preparations, the absorbances were determined at a heme protein concentration of 0.25–1.3 μM. In this range of concentration, the absorbances exhibited a linear relationship with either protein or heme protein P-450 concentration.

Results

Substrate-Induced Difference Spectra. The substrate-induced difference spectra observed with various oxygenated cholesterol derivatives obtained with the acetone-dried powder preparations are given in Table I. As may be seen, type I difference spectra were observed with 20β-hydroxy-20-isocholes-

[2] Submitted for publication.

TABLE I: Substrate-Induced Difference Spectra and Affinity Relationships with Acetone-dried Powder Preparations Observed with Cholesterol Oxygenated Derivatives.[a]

Prepn	Substrates	$\Delta A \times$ (mg of Protein/ml)$^{-1}$
1	20α-Hydroxycholesterol	+0.0188
1	20β-Hydroxy-20-isocholesterol	−0.0135
1	(22*R*)-22-Hydroxycholesterol	+0.0114
1	(22*S*)-22-Hydroxycholesterol	+0.0138
1	25-Hydroxycholesterol	−0.0092
1	20α,21-Dihydroxycholesterol	+0.0138
1	(22*R*)-20α,22-Dihydroxycholesterol	−0.0175
1	(22*S*)-20α,22-Dihydroxycholesterol	+0.0188
1	22-Oxocholesterol	+0.0180
1	20α-Hydroxy-22-oxocholesterol	−0.0037
1	**20α + 20β**	+0.0166
1	**20α + 25**	+0.0154
1	**20α + 20α,22-oxo**	+0.0142
1	**20,22R + 20,21**	−0.0154
1	**20,22R + 20,22S**	−0.0098
1	**20,22R + 22-oxo**	−0.016
2	**20α**	+0.015
2	**22R**	+0.010
2	**20,22R**	−0.0012[b]
2	**20α + 20,22R**	−0.0027[b]
2	**20,22R + 22R**	+0.0059

[a] The absorbance differences (ΔA) were given for the algebraic differences observed between the absorbances at 420 and 390 nm. Type I difference spectra are thus expressed as negative, while type II difference spectra appear as positive values. [b] These values were probably not significantly different from 0 because of the broadness of the observed difference spectra.

TABLE II: Substrate-Induced Difference Spectra and Affinity Relationships Observed with Partially Purified Heme Protein P-450 Preparations.

Prepn	Substrates	Extinction Coef ($cm^{-1}\ \mu M^{-1}$)
3	**20α**	+0.0591
3	**22R**	+0.0313
3	**20,22R**	0
3	**20α + 20,22R**	+0.0348
3	**22R + 20,22R**	+0.0226
4	**20α**	+0.0945
4	**22R**	+0.0558
4	**20,22R**	−0.0155
4	**20α + 20,22R**	+0.042
4	**22R + 20,22R**	+0.037
5	**20α**	+0.054
5	**22R**	+0.032
5	**20,22R**	−0.008
5	**20α + 20,22R**	+0.008
5	**22R + 20,22R**	+0.014
6	**20α**	+0.0116
6	**22R**	−0.0256
6	**20,22R**	−0.0713
6	**20α + 20,22R**	−0.0279
6	**22R + 20,22R**	−0.0276
7	**20α**	+0.0181
7	**22R**	−0.0422
7	**20,22R**	−0.116
7	**20α + 20,22R**	−0.0506
8	**20α**	+0.0096
8	**22R**	−0.0434
8	**20,22R**	−0.105
8	**20α + 20,22R**	−0.0349
9	**20α**	0[a]
9	**22R**	−0.0438
9	**20,22R**	−0.0875
9	**20α + 20,22R**	−0.0328
10	**20α**	0[a]
10	**22R**	−0.033
10	**20,22R**	−0.0917
10	**20α + 20,22R**	−0.0417
10	**20α + 22R**	−0.0313
11	**20α**	0[a]
11	**22R**	−0.0421
11	**20,22R**	−0.0816
11	**20α + 20,22R**	−0.0342

[a] The difference spectra of the 20α with these preparations were variable; in some experiments broad (type I) spectra were observed possessing extinction coefficients of the order of −0.03. See explanation to Table I for the meaning of the algebraic sign.

terol, 25-hydroxycholesterol, (22*R*)-20α,22-dihydroxycholesterol, and 20α-hydroxy-22-oxocholesterol, while type II difference spectra were obtained with 20α-hydroxycholesterol, (22*R*)-22-hydroxycholesterol, (22*S*)-22-hydroxycholesterol, 20α,21-dihydroxycholesterol, (22*S*)-20α,22-dihydroxycholesterol, and 22-oxocholesterol. The affinity relationship between these sterols was studied (at equimolar concentrations) by adding the substrates exhibiting type I to those showing type II difference spectra and noting the change in absorbance. As may be seen from Table I, **20β**, **25**, and **20α,22-oxo** caused only a relatively slight change in the absorbance of **20α** indicating that the 20α-hydroxycholesterol, at the concentrations used, had a higher affinity than these other sterols. Similarly, **20,22R** had a higher affinity than **20,21** and **22-oxo**, but probably only a slightly larger affinity than **20,22S**. When (22*R*)-22-hydroxycholesterol was added to both cuvets, no difference spectra were observed with **20α**, **22S**, **20,22S**, and **22-oxo**. When (22*R*)-20α,22-dihydroxycholesterol was added to both cuvets, no difference spectra were observed with **25**, **20β**, and **20α,22-oxo**, indicating that **22R** and **20,22R**, with the acetone powder preparation 1, exhibited the highest affinities.

With the acetone-dried powder preparation 2, (22*R*)-20α,22-dihydroxycholesterol did not exhibit a substrate-induced spectrum, yet in its presence (at equimolar concentrations), **20α** did not produce a significant difference spectrum (Table I). As observed with preparation 1, when (22*R*)-22-hydroxycholesterol was placed in both cuvets, no difference spectrum was elicited by **20α**. **20,22R** and **22R** appeared to have similar affinities with the acetone-dried powders.

The order of sterol addition had no influence on the finally developed difference spectra. Thus, when 20α-hydroxycholesterol was first placed in the reference cuvet and the difference spectrum was allowed to develop fully (approximately

TABLE III: Substrate-Induced Spectra and the Enzymatic Conversion of Cholesterol to Pregnenolone.[a]

Expt No.	Prepn	Difference Spectra (cm^{-1} μM^{-1}) Induced by: 20α	22R	20,22R	Pregnenolone Formation from Cholesterol: nmoles min^{-1} (Heme Protein P-450)$^{-1}$
1	2	+0.1	+0.06	−0.029	0.67
2	3	+0.059	+0.023	0	0.36
3	4	+0.095	+0.056	0	2.3
4	5	+0.054	+0.032	0	0.3
5	6	+0.011	−0.026	−0.071	0.44
6	7	+0.018	−0.042	−0.115	1.09
7	8	+0.009	−0.042	−0.105	0.58
8	9	0[b]	−0.040	−0.090	1.36
9[c]	9	0	+0.036	−0.084	0.64
10	10	0[b]	−0.033	−0.092	2.2
11	11	0[b]	−0.042	−0.081	1.26

[a] The incubation volumes were 3–3.1 ml and the time of incubation was 15 min. The cholesterol concentrations in the incubation media were in expt 1–11: 20.4, 3.4, 3.0, 6.1, 4.3, 4.3, 4.3, 4.3, 22.2, 2.6, and 4.3 μM, respectively. The cholesterol radioactivity added in these experiments was 19.8, 211, 211, 211, 4.6, 4.6, 4.6, 4.6, 21.7, 211, and 4.6 million dpm, respectively. The heme protein P-450 concentrations in these experiments were 0.015, 0.031, 0.043, 0.033, 0.1, 0.083, 0.071, 0.043, 0.043, 0.040, 0.025 μM, respectively. The scintillation counting efficiency was varied between 26.3 and 54.5%. [b] See footnote to Table II. [c] This experiment was repeated after preparation 9 was kept frozen for 1 month.

6–15 min) and was then followed by the addition of the **20,22R**, a gradual decrease in the difference spectrum to the values given in Table I was observed. Similarly, the same final result was obtained when the (22*R*)-20α,22-dihydroxycholesterol was first placed in the reference cuvet: in this case, however, the difference spectrum of the **20,22R** did not significantly change after the addition of the **20α**. The dissociation constants (K_S) were determined with preparation 2 affording approximate values for the **22R** and **20α** of 0.4 and 2.0 μM, respectively, substantiating the observations presented in Table I. Because of the variability observed between the various heme protein P-450 preparations (to be described below), K_S values were not determined with the other preparations. This was not even possible, for example, when no substrate-induced spectra were observed at all. Rather, a rough idea on affinity relationships among the hydroxylated sterols was obtained from results determined after the simultaneous addition of combinations of the sterols. These are presented for **20α**, **22R**, and **20,22R** observed with the other partially purified heme protein P-450 preparations in Table II. It is apparent from this table that with the preparations made according to Jefcoate *et al.* (1970) (preparations 3–5), the 20α-hydroxycholesterol exhibited the highest difference extinction coefficients, in comparison to **22R** and **20,22R**, as was also the case with the acetone-dried powders (*cf.* Table I). The (22*R*)-20α,22-dihydroxycholesterol either exhibited no difference spectrum at all or a relatively small type I spectrum, while the **22R** elicited a type II difference spectrum. From the experiments in which combinations of the sterols were used, it appeared that with these preparations **20,22R** did not manifest a consistently higher affinity as compared to the **20α** (as was observed with the acetone-dried powder preparations 1 and 2): compare preparations 3 and 4 in which **20,22R** obliterated the 20α spectra by only 40–55%, while with preparation 5 **20,22R** inhibited the appearance of 85% of the **20α** spectrum. The (22*R*)-22-hydroxycholesterol, when added to both cuvets, inhibited the appearance of the 20α-hydroxycholesterol spectrum but not to as high an extent as was observed with the acetone-dried powders, indicating that the affinity differences between **22R** and **20α** were not as large with these preparations. With the partially purified heme protein P-450 preparations made according to the procedure of Schleyer *et al.* (1971)[2] (preparations 6–11), while **20,22R** exhibited a relatively high extinction coefficient, **20α**, which elicited either no spectrum at all or a relatively small type I or type II spectrum, inhibited the appearance of the **20,22R** spectrum by 55–68%. It is noteworthy that with these (Triton treated) preparations, the (22*R*)-22-hydroxycholesterol exhibited a type I difference spectrum. With these preparations, the 20α-hydroxycholesterol and the (22*R*)-22-hydroxycholesterol appeared to exhibit as high or higher binding affinity than **20,22R**.

Comparison of the Enzymatic Transformation of Cholesterol to Pregnenolone with the Substrate-Induced Spectra. This is given in Table III. As may be seen from this table, there appeared to be no consistent correlation between the type of spectrum or its extinction coefficient and the rate of pregnenolone formation from cholesterol. Thus, relatively high enzymatic rates were observed with preparations 4 and 10 (expt 3 and 10). Yet, no difference spectrum was induced with **20α** with preparation 4, while with preparation 10 a relatively large difference spectrum was observed. The converse relationship was found with **20,22R**.

Discussion

There did not appear to be a consistent correlation between the sterol structure and the type of substrate-induced spectrum observed. Thus, with the acetone-dried powder preparation, the two epimeric 20-hydroxycholesterol derivatives, the 20α-hydroxycholesterol and the 20β-hydroxy-20-isocholesterol exhibited opposite difference spectra of similar intensity: **20α**, type II; **20β**, type I. The same relationship was observed with the (22*R*)-20α,22-dihydroxycholesterol and the (22*S*)-20α,22-dihydroxycholesterol. However, the **22R**- and **22S**-hydroxylated cholesterol derivatives both exhibited type II difference spectra. The conversion of cholesterol to pregnenolone could not be consistently correlated with the observed extinction coefficients of any of the hydroxylated cholesterol derivatives. It is interesting that with the acetone-dried powders, a positive correlation was observed between the affinities of the (22*R*)-22-hydroxycholesterol, (22*R*)-20α,22-dihydroxycholesterol, and 20α-hydroxycholesterol and their enzymatic rates of conversion to pregnenolone (*cf.* Burstein and Gut, 1971) despite the lack of correlation with the extinction coefficients.[3] However, this correlation appeared to break down with some of the other heme protein P-450 preparations stud-

[3] A preliminary report on substrate-induced difference spectra and enzymatic activities of these hydroxylated cholesterol derivatives has appeared (Burstein *et al.*, 1971).

ied, again not allowing any broad correlation between enzymatic rates and the difference spectra.

In recent studies, Whysner *et al.* (1970), suggested, on the basis of observed type II difference spectra with 20α-hydroxycholesterol and its affinity constant, that the **20α** may be serving as an enzyme-bound intermediate in the conversion of cholesterol to pregnenolone. The observations described by us suggest that because the difference spectra may vary drastically between various preparations, both in respect to the magnitude and affinity, correlations between the spectra and mechanistic enzymatic projections be viewed with great caution. It appears from the studies reported here that broad generalizations regarding the meaning of the difference spectra are not justified, and that they, by themselves, could hardly serve as a guide in studies on enzyme separations. The studies of Jefcoate *et al.* (1970), for example, in which fractions were obtained that were enriched in the cholesterol side-chain cleavage activity and exhibited definite difference spectra with 20α-hydroxycholesterol, may represent only a special case, that depends very much upon the conditions used in the purification. Van Lier and Smith (1970) reported that, with their adrenocortical mitochondrial acetone-dried powder preparation, (22*R*)-20α,22-dihydroxycholesterol did not exhibit a substrate-induced spectrum. Again, it is clear from the data presented here that this can only be stated in reference to a particular preparation but not in general.

The question of whether type I or type II difference spectra elicited by these substrates represent heme protein P-450 preparations in different "spin states" cannot be answered from the studies presented here. The studies by Schleyer *et al.* (1971a,b), cast doubts on the validity of the interpretation of the spectral changes in terms of the spin state of heme protein P-450. Our findings that relatively strong interactions with sterols may occur despite the lack of significant spectral effects in the Soret region (*cf.*, for example, Table I, preparation 2) are intriguing and further question the true meaning of this physical method as a tool in studying heme protein P-450.

Unequivocal answers to the important questions of variations in the concentration of the endogenous cholesterol or of the presence of inhibitors (*cf.* Mason and Boyd, 1971) (or activators) in the various heme P-450 fractions cannot be given at this stage. These, of course, could affect the enzymatic activities reported and may require a modification of some of the conclusions arrived at by us. It would appear that until enzymic preparations of a high degree of purity become available, the study of the correlation of enzymatic activity with various physical properties of heme protein P-450 may remain open to numerous interpretations that are difficult to check.

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