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Ligand Interaction with Hemoprotein P-450

III. The Use of *n*-Octylamine and Ethyl Isocyanide Difference Spectroscopy in the Quantitative Determination of High- and Low-Spin P-450

C. R. E. JEFCOATE, R. L. CALABRESE, AND J. L. GAYLOR

Section of Biochemistry and Molecular Biology and The Graduate School of Nutrition, Cornell University, Ithaca, New York 14850

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SUMMARY

A simple method for the determination, by ligand binding to octylamine, of the absolute amounts of high- and low-spin forms of hemoprotein P-450 of rabbit liver microsomes is described. A correlation between octylamine binding to the two forms of oxidized P-450 and the binding of ethyl isocyanide to reduced P-450 has been observed, and advantages of the amine-binding method over the use of ethyl isocyanide are pointed out. In particular, octylamine binds to each species of P-450 to produce a distinct difference spectral minimum. We have derived a relationship for the calculation of the proportion of each form of P-450.

When the amine-binding method was used to determine the amounts of high- and low-spin forms of P-450, interesting quantitative changes were observed. Variations in the contents of the two forms in rabbit liver microsomes were noted following maintenance of rabbits on different control diets; dietary oxytetracycline, commonly used in commercial feeds, further increased the proportion of the low-spin form. Microsomal P-450 of bovine adrenal cortex is almost entirely in the low-spin form, while adrenal mitochondria contain both forms in nearly equal proportions.

Application of both the octylamine and ethyl isocyanide methods provides complementary data about P-450 in either oxidation state. A distinction between the two forms of oxidized P-450 in liver microsomes is maintained quantitatively during conversion to the reduced state.

INTRODUCTION

Ethyl isocyanide combines with two forms of reduced hemoprotein P-450 to produce spectral maxima at approximately 430 and 455 mμ (1). The intensities of the two spectral peaks are affected by many factors, such as changes in pH (2), and are particularly altered by various prior treatments of experimental animals. For example, at a fixed pH, the ratio of intensity at 455 mμ to that at 430 mμ is increased by prior treatment of rats with 3-methylcholanthrene (2). This procedure also alters P-450–dependent rates of drug metabolism (2, 3). Thus, the amounts of each form present are reflected in metabolic patterns. Although the use of ethyl isocyanide for these spectral measurements affords a simple assay of relative changes resulting from prior treatment with drugs,

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its value for the determination of the absolute amounts of the two forms of P-450 is limited in a number of ways.

Octylamine, on the other hand, combines with two forms of oxidized P-450 of rabbit liver microsomes to produce two superimposed difference spectra having, for type *a*, λ_{max} 427 mμ and λ_{min} 392 mμ (high-spin form); and, for type *b*, λ_{max} 432 mμ and λ_{min} 410 mμ (low-spin form) (4). The amount of each form may be altered by prior treatment of the rabbits (5). For example, treatment with 3-methylcholanthrene increases the specific content of the form of P-450 that exhibits the type *a* difference spectrum (5). Thus, the ratio of absorbance changes at 410 and 392 mμ produced in oxidized P-450 upon binding octylamine varies in a manner analogous to that reported for the absorbance ratio changes of reduced P-450 on binding to ethyl isocyanide.

This report describes the difference spectral technique using octylamine and a correlation of the spectra observed with octylamine and ethyl isocyanide. Furthermore, because binding of types *a* and *b* corresponds to interactions of octylamine with high- and low-spin forms of P-450, respectively (4, 5), a method for the calculation of the absolute amounts of the two forms is given. The application of this method provides some interesting variations in the amounts of the two forms of P-450 observed with liver microsomes from rabbits on various "control" diets. Finally, both ethyl isocyanide and octylamine spectral techniques were applied to P-450 in both mitochondria and microsomes from the bovine adrenal cortex. P-450 from this tissue is consequently compared with that from rabbit liver microsomes.

MATERIALS AND METHODS

Animals. Male rabbits of the New Zealand strain, weighing approximately 2 kg, were used for all experiments. The rabbits were fed Purina rabbit chow ("medicated") for at least 1 week before death unless otherwise indicated. All rabbits were fasted for 24 hr prior to death. 3-Methylcholanthrene was administered, where indicated, as a single dose (20 mg/kg) in corn oil intraperitoneally 4 days before death (5).

Preparation of microsomes. Liver microsomes were prepared as described by Mason *et al.* (6). In brief, the livers were removed from the rabbits and perfused with 0.25 M sucrose solution. The livers were then weighed, cut into small pieces, and transferred to a Waring Blendor containing a volume (in milliliters) of 0.25 M sucrose solution equal to twice the weight (in grams) of liver. The liver was then homogenized three times (15 sec each time), and the crude suspension was centrifuged at 700 × *g* (Servall centrifuge; *r* = 4.25-cm rotor, 2400 rpm) for 5 min. Centrifugation was then continued for 15 min at 10,000 × *g* (approximately 10,000 rpm) to remove mitochondria. The resulting supernatant fraction was then decanted and centrifuged again at approximately 105,000 × *g* (maximum force in the Spinco No. 30 rotor, 30,000 rpm) for 1 hr. The resulting supernatant fraction was discarded, and the microsomal pellets were collected and suspended in a volume of 0.1 M potassium phosphate buffer, pH 7.4, equal to that of the original 10,000 × *g* supernatant fraction. The suspension of microsomes was collected by further centrifugation at 105,000 × *g* for 1 hr. Microsomes were used immediately or stored for no more than a few days in a small volume of the 0.1 M phosphate buffer (pH 7.4, containing 50% glycerol) at −20° under a nitrogen atmosphere.

Analytical methods. Total P-450 was determined from the CO difference spectrum of a dithionite-treated sample, using a millimolar extinction coefficient for the absorbance difference between 450 and 490 mμ of 91 mM^{-1} cm^{-1} (7). Protein was estimated by the method of Lowry *et al.* (8), using bovine serum albumin as a standard.

For determination of high- and low-spin forms of P-450, microsomes were suspended carefully in the glycerol-phosphate buffer to yield a protein concentration of 2–3 mg/ml. All spectra were measured at room temperature in 1-cm cells in a Cary model 14 recording spectrophotometer equipped with a high-sensitivity slide wire and a high-intensity visible light source. Baselines were traced. Difference spectra were obtained by addition of microliter quantities of a solution of the

ligand to one of the P-450–containing suspensions, while an equal volume of buffer was added to the reference cuvette. Spectra were traced within a few minutes. Particular care was taken to adjust the pH of the *n*-octylamine solution to 7.4 before use. Concentrations of high- and low-spin forms were calculated as described below.

The final concentrations of the ligands used were: ethyl isocyanide, approximately 0.5 mM; and *n*-octylamine, 1.0 mM. For ethyl isocyanide spectra, 10 μl of a solution of 0.09 M ethyl isocyanide were added to 1.8 ml of suspension; P-450 was reduced by adding a few grains of solid dithionite to each cuvette immediately before measurement.

Chemicals. All common chemicals were of the highest grade of purity available commercially. Ethyl isocyanide was the generous gift of Dr. M. Anders of Cornell University. Octylamine was obtained from the Aldrich Chemical Company.

RESULTS

Octylamine Difference Spectra of Oxidized P-450

Increasing concentrations of *n*-octylamine saturate first type *b* P-450 (low-spin form), having two binding constants of 0.005 and 0.034 mM, and then type *a* P-450 (high-spin form), having one binding constant, 0.3 mM (4). Thus, 1.0 mM octylamine is sufficient to saturate completely both forms of microsomal P-450. Accordingly, spectral measurements were carried out with this concentration of octylamine.

Difference spectra with octylamine were recorded using microsomes either from rabbits that were fed on commercial diets or from animals previously treated with 3-methylcholanthrene. The difference spectra of the two forms of P-450 are clearly differentiated (Fig. 1): type *a* has λ_{max} 427 mμ and λ_{min} 392 mμ, and type *b* has λ_{max} 432 mμ and λ_{min} 410 mμ. The relative proportions of these two forms can be calculated conveniently from either the standard curve (Fig. 2) or the equation shown in the legend to Fig. 2. Thus, as shown in Fig. 2, the $\Delta A_{410}:\Delta A_{390}$ ratio for liver microsomes obtained from rabbits on a control diet yields a ratio of type *a* to type *b* P-450 of approximately 0.25, while prior treatment with 3-methylcholanthrene on the same diet increases the ratio to approximately 1.0.

Correlation of Amine and Ethyl Isocyanide Spectra

Since both the *a* and *b* species of P-450 form pairs of ethyl isocyanide complexes of

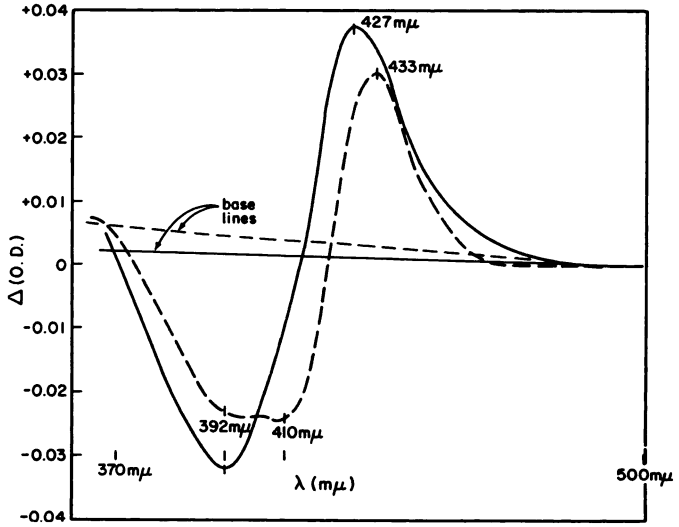

FIG. 1. *n-Octylamine difference spectra, 500–370 mμ, for 3-methylcholanthrene-induced* (——) *and control* (– – –) *rabbit liver microsomes*

Difference spectra were recorded with microsomal suspensions in glycerol-phosphate buffer. The concentration of octylamine was 1.0 mM.

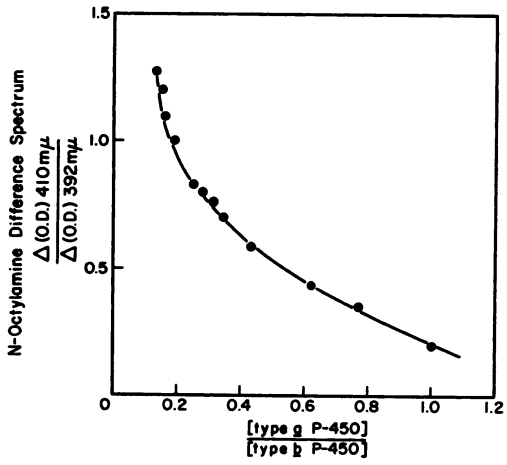

FIG. 2. *Standard curve relating* ΔA_{410}:ΔA_{392} *ratio of n-octylamine difference spectrum to the ratio of amounts of each type of P-450 according to Eq. 1*

The points represent data obtained from 12 samples of rabbit liver microsomes as described in Tables 1, 3, and 4.

$$\frac{[\text{type } a]}{[\text{type } b]} = \frac{\Delta A_{392} - x\Delta A_{410}}{z[\Delta A_{410} - y\Delta A_{392}]} \quad (1)$$

$$\Delta A_{392} = A_{392} - A_{500}$$

$$\Delta A_{410} = A_{410} - A_{500}$$

$$x = \frac{\Delta\epsilon_{b(-392)}}{\Delta\epsilon_{b(-410)}}; \quad y = \frac{\Delta\epsilon_{a(-410)}}{\Delta\epsilon_{a(-392)}}; \quad z = \frac{\Delta\epsilon_{a(-392)}}{\Delta\epsilon_{b(-410)}}$$

Calculated constants for rabbit liver microsomes (4) are

$$x = 0.4; \quad y = -0.15; \quad z = 2.5 \quad (1a)$$

reduced P-450 that exhibit difference maxima at approximately 430 and 455 mμ, a corresponding relationship between spectral ratios and type *a* and type *b* contents exists for P-450–ethyl isocyanide spectra under uniform assay conditions. Thus, in Fig. 3, correlation of the spectral ratios is shown for ethyl isocyanide and octylamine difference spectra obtained with samples containing different proportions of the *a* and *b* species of P-450. These samples were prepared by mixing liver microsomes obtained from rabbits fed a control diet (more than 80% of type *b* P-450) with liver microsomes from rabbits treated with 3-methylcholanthrene (approximately equal quantities of type *a* and type *b*). Both sets of spectral ratios gave linear correlations with the percentage com-

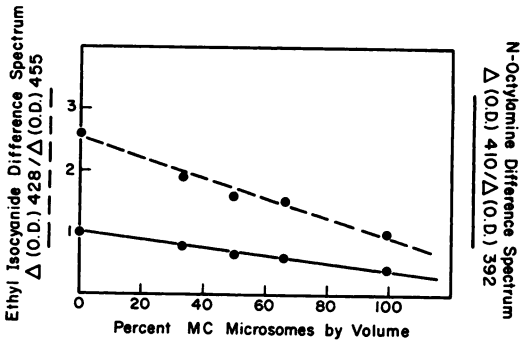

FIG. 3. *Change in spectral ratios related to relative amounts of types b and a of P-450 by two different assays*

Two suspensions of microsomes were used in this experiment. Microsomes were obtained from controls and rabbits treated with 3-methylcholanthrene (MC). These suspensions were combined in various proportions by volume to yield various concentrations of types *a* and *b* P-450. Difference spectra were taken on the microsomal suspensions (in 50% glycerol–0.1 M phosphate buffer, pH 7.4).

position of the types of P-450 (type *a* increases in proportion to the percentage of microsomes derived from methylcholanthrene-treated animals). Thus, over this limited range, a linear correlation between the two assay methods was observed.

A slightly curvilinear correlation was observed when the ratio of the two forms was substantially greater (Fig. 4). That is, in contrast to the limited set of data shown as a linear relationship in Fig. 3, in which the extremes in ΔA_{428}:ΔA_{455} were 0.5–2.5, the results shown in Fig. 4 include the extremes of experimental samples measured by both spectral methods in this laboratory, in which ΔA_{428}:ΔA_{455} varied from about 0.5 to 4.3. Departures from linearity were observed when the ratio was increased from 2.5 to 4.0. Widely differing proportions of the two forms of P-450 were present (see Table 4).

Even within a very narrow range of concentrations of P-450 types *a* and *b* in different individual samples (not mixtures), the correlation between the two spectral methods was close enough to predict the ethyl isocyanide difference spectrum accurately from results with the amine (Table 1). Thus, in all cases there was a close correlation between the ratios obtained by each method.

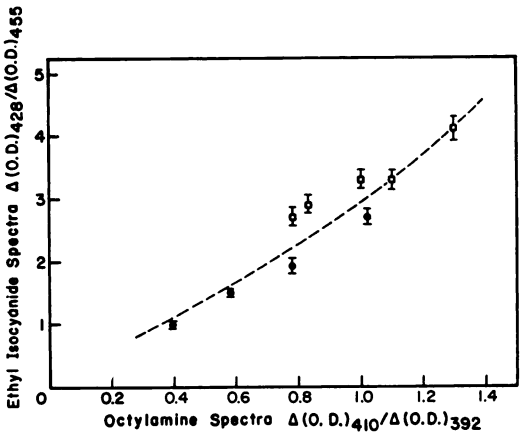

FIG. 4. *Correlation of octylamine and ethyl isocyanide difference spectra*

The samples were obtained from 3-methylcholanthrene-treated rabbits (●) and from control rabbits fed the Agway diet V (Table 4) (□). Unlike the experiment recorded in Fig. 3, mixtures were not prepared; individual values are reported. Ranges of variation in the values obtained for the ethyl isocyanide spectra are indicated.

TABLE 1

Correlation of octylamine and ethyl isocyanide difference spectra for rabbits fed various diets

The origins of the amine values are reported later in Table 4. The calculated value for ethyl isocyanide was obtained graphically (Fig. 4). The observed values for ethyl isocyanide were determined on the same samples of microsomes used for amine spectroscopy.

Amine difference spectrum (ΔA_{410}: ΔA_{392})	Ethyl isocyanide difference spectrum (ΔA_{428}:ΔA_{455})	
	Calculated	Observed
0.83	2.4	2.9
1.1	3.3	3.3
0.78	2.2	2.7
1.0	3.0	3.3
1.3	4.1	4.1

A mathematical correlation between ethyl isocyanide and octylamine difference spectra was obtained by substituting the following parameters for ethyl isocyanide spectra in Eq. 1 (Fig. 2) (392 mμ is replaced by 455 mμ, and 410 mμ by 428 mμ).

$$x = 0.2; \quad y = 0; \quad z = 0.9 \quad (1b)^1$$

[1] Equation 1a appears in the legend to Fig. 2.

TABLE 2

Percentage of type a of P-450 calculated from octylamine and ethyl isocyanide difference spectra

The calculation is described in the text.

Ethyl isocyanide method		Octylamine method	
Spectral ratio (ΔA_{428}:ΔA_{455})	Percentage of type *a* P-450	Spectral ratio (ΔA_{410}:ΔA_{392})	Percentage of type *a* P-450
	%		%
1	47.0	0.36	42.5
1.5	34.3	0.55	32.4
2	24.8	0.71	24.8
2.5	18.0	0.87	18.7
3	13.0	1.1	13.0
3.5	9.9	1.15	12.3
4	5.7	1.27	11.5

These values gave the best fit to the octylamine and ethyl isocyanide spectra as described in Table 1 and Figs. 3 and 4.

The accuracy of the assignment of coefficients then was tested (Table 2). A series of selected ethyl isocyanide spectral ratios was related to the percentage of type *a* P-450 by means of Eq. 1b. The octylamine spectral ratio corresponding to each ethyl isocyanide ratio was then determined from the curve shown in Fig. 4. The percentages of type *a* P-450 derived from these octylamine spectral ratios by means of Eq. 1a (Fig. 2) were very close to those derived by Eq. 1b. Deviation was detected only when the concentration of type *a* P-450 was rather low. Thus, the ethyl isocyanide spectra can be closely described by the two forms of P-450 related by Eq. 1b.

Applications of the Octylamine Difference Spectral Technique

Effect of diet. During this study and other studies in this laboratory (4, 5), rabbits were fed a commercial stock diet, Purina rabbit chow ("Medicated"). Occasionally, when male rabbits of the same age and strain were obtained from other workers in this department, varied proportions of types *a* and *b* P-450 in liver microsomes were observed. After we were satisfied that the variations could not be ascribed to experimental error they were traced to the use of different "control" diets.

To establish the effect of diet, and specifically how dietary antibiotics (of common use in commercial feeds) might affect liver P-450, rabbits were fed for various lengths of time on the Purina rabbit chow diet (containing a low level of oxytetracycline added by the manufacturer). Total hepatic microsomal P-450 was then assayed, and the relative amounts of the two types of P-450 were estimated by the octylamine difference spectral technique (Table 3). The ΔA_{410}:ΔA_{392} ratio obtained from the *n*-octylamine difference spectra increased sharply with exposure to the dietary antibiotic, indicating that the relative amount of P-450 type *b* had increased. The extinction coefficient for the P-450–CO complex (7) suggested that the specific content of P-450 had decreased somewhat with exposure to the diet. However, this apparent decrease may be attributed partially to a lower extinction coefficient at 450 mμ observed for the type *b* P-450–CO complex, which has previously been reported (4, 5).

The effect of oxytetracycline was verified by adding the antibiotic to two widely different control diets. As shown in Table 4, the addition of oxytetracycline to a synthetic diet prepared from purified ingredients also caused a decrease in the total content of P-450, with a shift to a much higher contribution of the type *b* P-450 hemoprotein (diet II as opposed to I). The magnitudes

TABLE 3

Effect of dietary oxytetracycline on relative amounts of type b and type a P-450

Rabbits were fed Purina rabbit chow (containing oxytetracycline added by the manufacturer). *n*-Octylamine difference spectra were measured on two solutions of the microsomes after various intervals of feeding; protein content was 2–3 mg/ml (in 50% glycerol-phosphate buffer, pH 7.4) as described in MATERIALS AND METHODS. The results are the averages of duplicate values from two separate experiments.

Length of feeding	Spectral ratio (ΔA_{410}:ΔA_{392})	P-450 content
		mμmoles/mg protein
0 days	0.59	1.31
3 days	0.71	1.16
1 week	0.79	1.04
6 weeks	0.92	0.90

TABLE 4

Effect of different diets on relative amounts of type a and type b P-450 as determined by n-octylamine difference spectra

Except as footnoted, each value is the average of data obtained from five rabbits in two separate experiments. The animals were fed the indicated diet for 6 weeks.

Origin	Diet[a]	Oxytetracycline	P-450 content	Amine difference spectrum	
				ΔA_{410}:ΔA_{392}	[Type *a*]:[type *b*]
			mμmoles/mg protein		
Synthetic	I	−	1.32	0.83 ± 0.05[b]	0.25
Synthetic	II	+	0.87	1.1 ± 0.10	0.16
Purina	III	−	1.22	0.78 ± 0.05	0.31
Purina	IV	+	0.93	1.0 ± 0.09	0.19
Agway	V	−		1.3 ± 0.12	0.13
	I[c]		0.79	1.3	0.13

[a] Diets: I, synthetic rabbit diet; General Biochemicals, catalog No. 107970; pelleted to 3/16 inch; fed ad libitum. II, diet I, with oxytetracycline (Pfizer Laboratories) added at the concentration contained in Purina rabbit chow ("medicated"); fed ad libitum; III, Purina rabbit chow; fed 150 g/day;[d] IV, Purina rabbit chow ("medicated"), oxytetracycline added by manufacturer; fed 150 g/day;[d] V, Agway diet, no antibiotic added; fed 150 g/day.[d]

[b] Standard deviations; five samples each.

[c] Weanling rabbits fed diet I for approximately 15 weeks; four rabbits in two separate experiments.

[d] Equal to daily food consumption in group I.

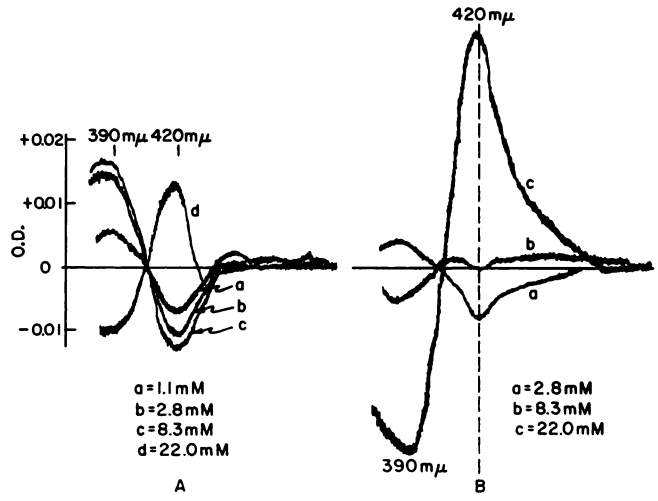

FIG. 5. *Effect of diet on phenobarbital difference spectra*

Phenobarbital (at neutral pH in 50% aqueous methanol) was added to the sample cuvette; an equal volume of 50% aqueous methanol was added to the reference cuvette. Microsomes were suspended under the conditions described for Fig. 1. Liver microsomes were obtained from rabbits fed a diet containing oxytetracycline (see Table 4) for either 6 weeks (A) (protein, 6.5 mg/ml) or 3 days (B) (protein, 5 mg/ml). The specific P-450 content was the same in both parts A and B.

were approximately equal to the effect of addition of oxytetracycline to Purina rabbit chow (diet IV vs. III). Therefore, the changes appear to be ascribed to the addition of the antibiotic regardless of the basal diet.

Variations in specific content and relative amounts of each type of P-450 were observed when the diets contained no oxytetracycline (Table 4, diets I, III, V, and I^c). For example, the liver microsomes from rabbits fed diet V had the lowest ratio of type *a* to type *b* species of P-450. The difference spectrum with octylamine almost approached that of the pure type *b* (low-spin) P-450 complex (4).

In all previous experiments, male rabbits 13–15 weeks old had been obtained from a supplier that had fed the does a commercial stock diet, Wayne rabbit pellets. Their young had had access to this diet before and after weaning. We obtained weanling rabbits 6 weeks of age from the same supplier. The young rabbits were trained to eat the synthetic diet of group I of Table 4. After 15 weeks, when the rabbits were the same age as those shown in Table 4, assay for types *a* and *b* species of P-450 showed a very low ratio of type *a* (high-spin) to type *b* (low-spin) P-450 (diet I^c of Table 4). Because this diet contained only synthetic or highly purified materials, the further drop in the ratio from 0.25 to 0.13 for rabbits fed diets I and I^c, respectively, indicated that induction of the high-spin form of P-450 (type *a*) may be ascribed to dietary constituents in the commercial Wayne rabbit pellets. Clearly, in the absence of induction, the type *a*, or high-spin form, of P-450 was scarcely present. Variations in food consumption between rabbits in the different groups was eliminated by limiting all groups to a consumption of 150 *g*/day. This consumption approximated the ad-libitum feed intake of group I.

The discovery of these dietary effects has helped us to explain a major anomaly in spectral studies reported earlier by us (4) and others (9, 10). Figure 5 shows difference spectra obtained by adding phenobarbital to liver microsomes obtained from rabbits fed the purina diet containing oxytetracycline for 3 days and 6 weeks. Phenobarbital binds to type *b* P-450 at low concentrations, producing a difference minimum at 420 mμ, while, at higher concentrations, this difference spectrum is first removed and then inverted by binding to type *a* P-450,

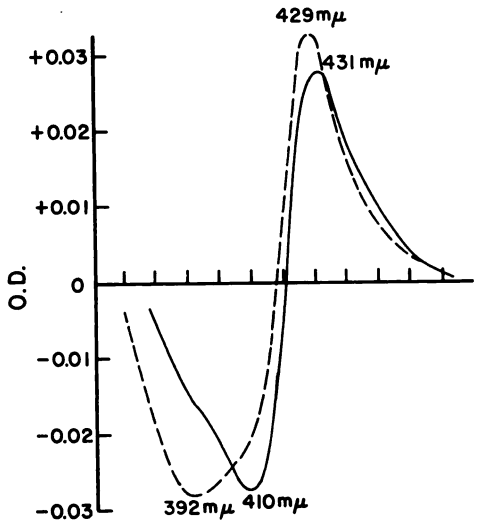

FIG. 6. *Binding of octylamine to P-450 of bovine adrenal microsomes and sonicated mitochondria*

Microsomes (——): carbon monoxide difference spectrum of reduced P-450 was 0.10 absorbance unit. Sonicated adrenal mitochondria (- - -); carbon monoxide difference spectrum of reduced P-450 ($A_{450} - A_{490}$) was 0.12. The concentrations of octylamine were 3.0 and 2.7 mM, respectively.

which produces a difference maximum at 420 mμ. Thus, the phenobarbital-induced difference spectra, which are generally referred to as typical type I spectra, may be readily varied by dietary alterations that provide the animals with substances evoking changes in the proportions of two forms of P-450 that have been detected by the amine method in the microsomal samples considered in Tables 3 and 4.

P-450 in adrenal cortex. Bovine adrenal cortex mitochondria, submitochondrial particles, and microsomes were prepared according to Harding *et al.* (11), and the spectral changes produced by *n*-octylamine were examined. The difference spectrum obtained from adrenal microsomes with *n*-octylamine (Fig. 6) showed the characteristics (λ_{max} 432, λ_{min} 410 mμ, with $\Delta A_{410}:\Delta A_{392}$ 1.5–2.0) of the pure type *b* P-450. However, in contrast to the type *b* P-450 in rabbit liver microsomes (4), only a single, rather weak, binding constant was observed (K = 1.0 mM).

Adrenal mitochondria showed the same type *a* and type *b* difference spectra (Fig. 6) found in liver microsomes; however, the spectral ratio corresponded to nearly equal proportions of the two forms. Binding constants for octylamine showed sequential combination, first to type *b*, then to type *a* (type *b*, K = 0.4–0.6 mM; type *a*, K = 1.8–2.5 mM).

Measurement of ethyl isocyanide difference spectra was attempted with reduced P-450 of adrenal microsomes. The microsomes contained P-420, and the measurements failed. For adrenal mitochondria (whole and sonicated), under the conditions reported for liver microsomes, the difference maximum at 455 mμ was only about 25% of that at 428 mμ.

DISCUSSION

The advantages of octylamine difference spectroscopy are several. The main advantage derives from the simple demonstration of octylamine binding to two distinct forms of oxidized hemoprotein P-450. The *n*-octylamine complexes of both forms show similar spectra, and difference minima correspond directly to the original, distinct Soret peaks (Fig. 7). The extinction coefficients of these complexes have been calculated: type *a*, $\Delta\epsilon_{392}$ = 65 mM^{-1} cm^{-1}; type *b*, $\Delta\epsilon_{410}$ = 25 mM^{-1} cm^{-1} (4). Thus, effects that alter the proportions are measured directly. The high- and low-spin forms of oxidized P-450 are reduced to forms that are indistinguishable in the absence of added ligand, such as ethyl isocyanide. Because the proportions of ethyl isocyanide complexes absorbing at 428 and 455 mμ vary according to prior treatment of the animal, high- and low-spin oxidized P-450 must yield different proportions of the two species in the reduced state.

Other disadvantages of ethyl isocyanide difference spectroscopy may be important. The compound is quite volatile, and in aqueous solution it may exist in varying concentrations after storage. It has an intensely distasteful and persistent odor. It is not available commercially, and synthesis requires proper handling of large quantities of cyanide. Spectra of the hemoprotein complex are influenced by pH; spectral properties in the amine method are independent of pH in the range of pH 6.5–8.0, provided that saturating amounts of unprotonated amine

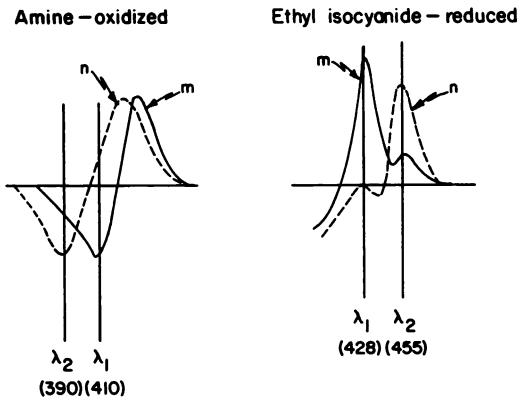

FIG. 7. *Two difference spectral techniques illustrated simply*

Two species, m and n, that are present at concentrations of C_m and C_n, respectively, react with a ligand to produce overlapping difference spectra; the change in optical density (ΔA) at two wavelengths, λ_1 and λ_2, is given as

$$\Delta A_{\lambda_1} = C_m \cdot \Delta\epsilon_{m\lambda_1} + C_n \cdot \Delta\epsilon_{n\lambda_1} \quad (2)$$

$$\Delta A_{\lambda_2} = C_m \cdot \Delta\epsilon_{m\lambda_2} + C_n \cdot \Delta\epsilon_{n\lambda_1} \quad (3)$$

($\Delta\epsilon_{m\lambda_1}$, etc., represents the extinction change for species m at wavelength λ_1, etc.) Then, dividing Eq. 2 by Eq. 3 yields

$$\frac{\Delta A_{\lambda_1}}{\Delta A_{\lambda_2}} = \frac{a(1 + bR)}{R + c} \quad (4)$$

where

$$R = \frac{C_n}{C_m}; \quad a = \frac{\Delta\epsilon_{m\lambda_1}}{\Delta\epsilon_{n\lambda_2}}; \quad b = \frac{\Delta\epsilon_{n\lambda_1}}{\Delta\epsilon_{m\lambda_1}}; \quad c = \frac{\Delta\epsilon_{m\lambda_2}}{\Delta\epsilon_{n\lambda_2}}$$

Rearranging and solving for R, the ratio of concentrations (i.e., C_n/C_m), the solved equation becomes

$$R = \frac{a(\Delta A_{\lambda_2} - c/a\Delta A_{\lambda_1})}{\Delta A_{\lambda_1} - a \cdot b\Delta A_{\lambda_2}} \quad (5)$$

Then

$$\frac{C_n}{C_m} = \frac{\Delta A_{\lambda_2} - x \cdot \Delta A_{\lambda_1}}{z(\Delta A_{\lambda_1} - y \cdot \Delta A_{\lambda_2})} \quad (6)$$

where

$$x = \frac{c}{a} = \frac{\Delta\epsilon_{m\lambda_2}}{\Delta\epsilon_{m\lambda_1}}; \quad y = a \cdot b = \frac{\Delta\epsilon_{n\lambda_1}}{\Delta\epsilon_{n\lambda_2}};$$

$$z = \frac{1}{a} = \frac{\Delta\epsilon_{n\lambda_2}}{\Delta\epsilon_{m\lambda_1}}$$

With substitution of type a of P-450 for n and of type b of P-450 for m (as shown in this figure), Eq. 7 results.

$$\frac{[\text{type } a]}{[\text{type } b]} = \frac{\Delta A_{(392)} - x \cdot \Delta A_{(410)}}{z(\Delta A_{(410)} - y\Delta A_{(392)})} \quad (7)$$

where

$$x = \frac{\Delta\epsilon_{b(392)}}{\Delta\epsilon_{b(410)}}; \quad y = \frac{\Delta\epsilon_{a(410)}}{\Delta\epsilon_{a(392)}}; \quad z = \frac{\Delta\epsilon_{a(392)}}{\Delta\epsilon_{b(410)}}$$

Obviously, Eq. 7, derived rigorously, is identical with Eq. 1 of Fig. 2, which was obtained experimentally. A similar calculation may be carried out for ethyl isocyanide.

are added (4). Reduction with sodium dithionite is of limited value, since the absorbance at the 455 mμ peak diminishes with time, possibly because of peroxidation of the P-450. Thus, reduction must be performed very carefully to avoid alteration of the hemoprotein. Finally, oxidized P-420 does not combine significantly with 1 mM solutions of *n*-octylamine (4); ethyl isocyanide combines with P-420 to yield an interfering absorption spectrum.

The bases of the two spectral methods may be quite different. In the oxidized state, differences in the two species of P-450 derive from the two spin states of iron. If models of reduced forms of P-450 are valid (12, 13), differences in the reduced P-450–ethyl isocyanide complexes derive from the association-dissociation reactions of porphyrin groups, and these reactions have no established correlation with the spin characteristics of the hemoprotein in the oxidized state.

However, the difference spectra of ethyl isocyanide complexes of reduced P-450 correlate well with estimates of the relative proportions of types *a* and *b* P-450 obtained by octylamine difference spectroscopic examination of oxidized P-450 (Table 1 and Figs. 3 and 4). Thus, complementary results may be obtained for both the oxidized and reduced states. The correlation found between the two forms in liver microsomes has been maintained upon reduction. This implies that the relative amounts of types *a* and *b* of P-450 are not significantly determined by reversible binding of endogenous substrates, because addition of these sub-

strates does not produce the changes that may be observed in the reduced state of P-450. The same point is supported by our previous observation (4) that not enough inducer (3-methylcholanthrene) is retained by the microsomes to account for the relative amounts of types *a* and *b* P-450 observed after treatment of the rabbits with 3-methylcholanthrene. Furthermore, the sequential binding of octylamine to type *b*, followed by saturation of type *a* P-450, rules out a simple equilibrium between the two forms. An alternative explanation recently was offered by Schenkman *et al.* (14).

By contrast, endogenous steroid substrates or other cellular components may play an important role in producing spectral changes in the *oxidized* state of P-450 in adrenal mitochondria. Type *a* P-450, which is found extensively in the *oxidized* state, seems to be absent from the ethyl isocyanide–*reduced* P-450 complex. Therefore, the correlation between the two methods could indicate the significance of such changes between the oxidized and reduced states of P-450. However, we emphasize that ethyl isocyanide spectra may be much more sensitive to the membrane environment of P-450. The range of means shown in Fig. 4 probably arises from a microenvironmental effect (possibly lipid content) on ethyl isocyanide spectra. Sensitivity to solvent polarity, ionic strength, and pH has been demonstrated (1–3). Octylamine spectra, however, should be relatively insensitive to environmental factors. Thus, the spectra and parameters of Eq. 1a calculated for amine binding to P-450 of bovine adrenal mitochondria were close to those for P-450 in rabbit liver microsomes.

The results shown in Table 3 indicate that orally administered oxytetracycline had a profound effect on microsomal P-450 even when given in low doses for only 1 week. This regimen increased the proportion of P-450 type *b*, with an apparent corresponding decrease in total P-450 content relative to animals not treated with the antibiotic. In contrast, prior treatment with either 3-methylcholanthrene or phenobarbital caused an increase in the specific content of P-450 (5). The data in Table 4 show that this effect was indeed due to oxytetracycline and not to other constituents in the diet, as administration of a totally different control diet, with oxytetracycline added, showed that the antibiotic had the same effect.

From the investigation of the effect of various "control" diets, some evidence for the induction of type *a* P-450 by commonly used dietary materials is apparent. For example, when the rabbits were fed the purified diet (diet I of Table 4) for 6 weeks, the content of type *a* P-450 was considerably higher than in the group fed the diet from weaning for 15 weeks (I^c of Table 4). Thus, previous commercial diets must have had a residual influence on the proportion of type *a* P-450 content in microsomes. It is noteworthy that Levin and Kuntzman (15) have found that in rats the form of P-450 induced by 3-methylcholanthrene [high-spin or type *a* (4)] has a biological half-life approximately 6 times as long as that of the low-spin form. Thus, carry-over of type *a* P-450 from a previous dietary treatment is quite possible.

This evidence suggests that in experimental work the following protocols should be noted carefully: (a) the diets the experimental animals are, and have been, fed; (b) treatment of commercially grown animals with antibiotics in their diet, drinking water, or as medication; and (c) the length of time the various diets are administered. Many apparent anomalies or discrepancies may be ascribed to hitherto undetected effects of "control" diets (e.g., Fig. 5).

Recently, Wilson *et al.* (16) and Whysner *et al.* (17) pointed out the relationship between two steroid-binding forms of P-450 in adrenal mitochondria and the two different spin states of P-450. Thus, the demonstration of both high- and low-spin forms of P-450 in adrenal mitochondria by the amine-binding method illustrates the ease of their measurement (Fig. 6). Differences in the rates and selectivity of metabolism of testosterone by different forms of liver microsomal P-450 (18), now known to be high- and low-spin forms (4, 5), suggest that adrenal mitochondrial steroid metabolism may also be related to different amounts of these two species.

The metabolic importance of measure-

ments of absolute amounts of high- and low-spin forms of P-450 has been established (3, 18). Amine difference spectroscopy affords a simple method that appears suited for wide ranges of contents of P-450 and samples from different sources.

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