Biochemical Pharmacology, Vol. 21, pp. 2577–2590. Pergamon Press, 1972. Printed in Great Britain.

SPECIES DIFFERENCES AND DRUG METABOLISM*

E. J. FLYNN,† M. LYNCH and V. G. ZANNONI

Department of Pharmacology, New York University, School of Medicine, New York, N.Y. 10016, U.S.A.

(*Received* 2 *February* 1972; *accepted* 28 *April* 1972)

Abstract—In this report three types of drug oxidation reactions were studied in mice, rats and guinea pigs, e.g. aminopyrine *N*-demethylation, *p*-nitroanisole *O*-demethylation and aniline hydroxylation. The studies *in vitro* show that no species emerged as a rapid or slow metabolizer of these prototype drug substrates. The determination of the activity and quantity of liver microsomal electron transport components indicate that cytochrome P-450 and NADPH cytochrome P-450 reductase are important factors in determining the activity of overall drug oxidation. However, the level of cytochrome b_5 and activity of NADPH cytochrome *c* reductase as well as the apparent affinity of the microsomal system for NADPH could not account for the observed differences in drug metabolism.

A study of the binding of drug substrates to cytochrome P-450 indicates that there are qualitative differences in this cytochrome which may contribute to species variation in drug metabolism. Species differences in cytochrome P-450 are not apparent in the reduced cytochrome P-450-CO complex or the typical type I (aminopyrine) and type II (aniline) substrate-cytochrome P-450 binding spectra. However, prolonged treatment of microsomes with aminopyrine or aniline results in an atypical type Ia or type IIa spectrum which has a maximum absorption at different wavelengths in various species.

Studies employing phenobarbital or 3-methylcholanthrene to induce individual electron transport components indicate that *N*-demethylase and *O*-demethylase activities are dependent upon the level of cytochrome P-450 and that aniline hydroxylase activity is dependent upon the level of cytochrome P-450 reductase activity.

SPECIES differences in the rate of metabolism of drugs have been demonstrated for many compounds which have a wide spectrum of pharmacological activity.[1–13] These differences can have profound effects on drug response in various species and, in fact, make it very difficult to extrapolate animal data to man.

The data presented are concerned with attempts to determine which liver microsomal electron transport components may be responsible for the observed differences in typical drug oxidation activities such as *O*-demethylase, *N*-demethylase and aniline hydroxylase in mice, rats and guinea pigs. The experiments *in vitro* presented include the determination of *p*-NO_2 anisole *O*-demethylase, aminopyrine *N*-demethylase and aniline hydroxylase activities; the determination of the quantity of liver microsomal electron transport components such as cytochrome P-450, cytochrome b_5 and NADPH cytochrome P-450 reductase activity; and the determination of

* This study was supported by Grant GM 17184 from the National Institutes of Health, United States Public Health Serivce and Grant GM 01447 from the National Institute of General Medical Sciences.

† This study was submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy in the Department of Pharmacology, New York University, New York, N.Y.

substrate–cytochrome P-450 binding spectra. A study of the activity and quantity of electron transport components in several species has allowed the assignment of various electron transport components and the elimination of others as important determinants in the degree of oxidation of aminopyrine, *p*-nitroanisole and aniline.

MATERIALS AND METHODS

Preparation of liver microsomes. Male albino guinea pigs (Hartley, 150–200 g body wt.), male mice (Swiss–Webster, 10–15 g) and male rats (Sprague–Dawley, 100–125 g) were decapitated, exsanguinated and their livers were removed and placed on ice. All following procedures were carried out at 4°. Homogenates (20%, w/v) were prepared in 1·15% KCl 0·02 M Tris, pH 7·4, with a Potter–Elvehjem homogenizer. The crude homogenate was spun at 15,000 *g* for 15 min. The supernatant fraction was spun at 100,000 *g* for 60 min at 2°. The microsomal pellet was suspended in 1·15% KCl 0·02 M Tris, pH 7·4, to one-half the original 15,000 *g* supernatant fraction volume; the protein concentration was in the order of 8–10 mg/ml. All enzyme assays were performed on freshly prepared microsomes.

p-*Nitroanisole* O-*demethylase activity.* Microsomal *p*-nitroanisole *O*-demethylase activity was determined by measuring the product of the reaction, *p*-nitrophenol, at 415 nm as previously described.[14,15]

The specific activity of *O*-demethylase is expressed as micromoles of *p*-nitrophenol formed per hour per 100 mg of microsomal protein at 27°.

Aminopyrine N-*demethylase activity.* Microsomal aminopyrine *N*-demethylase activity was determined by measuring the formaldehyde formed and measured at 412 nm by the colorimetric procedure of Nash, based on the Hantzsch reaction.[16]

The specific activity of *N*-demethylase is expressed as micromoles of HCHO formed per hour per 100 mg of microsomal protein at 27°.

Aniline hydroxylase activity. Microsomal aniline hydroxylase activity was determined by measuring the product, *p*-aminophenol, after formation of a complex with phenol reagent at 660 nm.[17]

The specific activity of aniline hydroxylase is expressed as micromoles of *p*-aminophenol formed per hour per 100 mg of microsomal protein at 27°.

NADPH cytochrome c *reductase activity.* Microsomal NADPH cytochrome *c* reductase activity was based on the absorbance of reduced cytochrome *c* at 550 nm according to the method of Williams and Kamin.[18]

The specific activity of NADPH cytochrome *c* reductase is expressed as micromoles of cytochrome *c* reduced per hr per 100 mg of microsomal protein at 27°.

NADPH cytochrome P-450 *reductase activity.* The determination of microsomal NADPH cytochrome P-450 reductase activity was based on the formation of reduced cytochrome P-450-CO by NADPH.[19,20] The reduction is performed in the presence of CO and followed in a Gilford 2000 recording spectrophotometer at 15°. The initial rate of the reaction was recorded (4 sec) and was linear with time and proportional to enzyme concentration. Under the conditions of the assay the molar extinction coefficient of cytochrome P-450-CO is 91 mM^{-1} cm^{-1}.

The specific activity of reductase activity is expressed as micromoles of cytochrome P-450 reduced per hr per 100 mg of microsomal protein at 27°. The Q_{10} of the reaction was 1·91.

Cytochrome P-450. The quantity of microsomal cytochrome P-450 was determined by the method of Omura and Sato.[21] Under the conditions of the assay the apparent molar extinction coefficient of the reduced P-450-CO complex is 91 mM^{-1} cm^{-1}.

The specific activity of cytochrome P-450 is expressed as micromoles of cytochrome P-450 per 100 mg of microsomal protein.

Cytochrome b_5 was determined by the method of Omura and Sato.[22] Under the conditions of the assay the molar coefficient of reduced cytochrome b_5 is 171 mM^{-1} cm^{-1}.

The specific activity of cytochrome b_5 is expressed as micromoles of b_5 per 100 mg of microsomal protein.

Substrate–cytochrome P-450 *binding spectra.* Aminopyrine (type I binding substrate) or aniline (type II binding substrate) was added to a microsomal suspension (2·0 mg protein/ml of 0·05 M Tris buffer, pH 7·4) as previously described.[23] The final concentration of substrate was 5·0 mM.

Phenobarbital and 3-methylcholanthrene induction. Groups of male, Swiss–Webster mice weighing 10–15 g were injected, i.p., with phenobarbital (sodium) or 3-methylcholanthrene. Phenobarbital was given at a dose of 50 mg/kg in 0·9% NaCl two times daily and 3-methylcholanthrene was given once a day at a dose of 80 mg/kg in corn oil. Livers were removed 18 hr after the last injection.

Protein was determined by the method of Lowry *et al.*[24] Crystalline bovine serum albumin, Fraction V, was used as a standard.

RESULTS

Species differences in activity of drug enzymes. Drug oxidation activities and the level and activity of electron transport components in groups of mice, guinea pigs and rats are given in Table 1. Although there was a 2- to 3-fold difference in enzyme

TABLE 1. ACTIVITY OF DRUG ENZYMES AND MICROSOMAL ELECTRON TRANSPORT COMPONENTS IN VARIOUS SPECIES*

Activity†	Mouse	Guinea pig	Rat
p-NO_2 anisole *O*-demethylase	40 ± 3·3	70 ± 3·8	20 ± 2·7
Aminopyrine *N*-demethylase	140 ± 8·3	70 ± 13	140 ± 11
Aniline hydroxylase	32 ± 1·3	10 ± 1·3	20 ± 1
NADPH cytochrome *c* reductase	1680 ± 85	2190 ± 150	1620 ± 37
NADPH cytochrome P-450 reductase	86 ± 11	36 ± 4	10 ± 1
Cytochrome P-450‡	0·6 ± 0·02	0·7 ± 0·04	1·0 ± 0·05
Cytochrome b_5‡	0·3 ± 0·01	0·4 ± 0·02	0·3 ± 0·08

* Mean ± S.E. of fifteen animals per group.
† Enzyme activity = micromoles of product formed per hr per g of microsomal protein at 27°.
‡ Quantity of cytochrome P-450 and cytochrome b_5 = micromoles per g of microsomal protein.

activities between the species, there was no apparent correlation between the amount of drug oxidation activity and the various species; e.g. the guinea pig, the species with the highest *O*-demethylase activity, did not have the highest *N*-demethylase and aniline hydroxylase activity. Furthermore, a higher level of cytcochrome P-450 in a

species did not necessarily lead to increased drug oxidation. For example, the rat which has the highest quantity of cytochrome P-450 has low *O*-demethylase activity, intermediate aniline hydroxylase activity and high *N*-demethylase activity. In addition, mice had the lowest quantity of cytochrome P-450 and the highest aniline hydroxylase activity. It should be noted that cytochrome b_5 and NADPH cytochrome *c* reductase levels showed little variation between the species. In contrast, there was marked variation in the level of NADPH cytochrome P-450 reductase. For example, the reductase activity in mice was eight times greater than in the rat. In addition, the variation found in this activity was great enough to be responsible for some of the differences observed in drug oxidation. For example, NADPH cytochrome P-450 reductase activity in the guinea pig and rat correlated well with their relative *O*-demethylase activity. However, mice which had a higher level of NADPH cytochrome P-450 reductase than guinea pigs had lower *O*-demethylase activity. Although the observed differences in the level of electron transport components could eliminate cytochrome b_5 and NADPH cytochrome *c* reductase as important determinants for overall drug oxidation, these studies could explain only some of the observed species variation.

The results on the determination of the apparent affinity constants (K_m) of NADPH and *p*-nitroanisole with rat, guinea pig and mouse microsomes are given in Table 2.

TABLE 2. APPARENT MICHELIS–MENTEN AFFINITY CONSTANTS (K_m) FOR NADPH CYTOCHROME *c* REDUCTASE AND *O*-DEMETHYLASE IN VARIOUS SPECIES*

Activity	Guinea pig (K_m)	Rat (K_m)	Mouse (K_m)
NADPH cytochrome *c* reductase†	$3{\cdot}0 \times 10^{-6}$ M	$6{\cdot}4 \times 10^{-6}$ M	$6{\cdot}5 \times 10^{-6}$ M
p-NO_2 anisole *O*-demethylase‡	$1{\cdot}8 \times 10^{-4}$ M	$1{\cdot}7 \times 10^{-3}$ M	$1{\cdot}6 \times 10^{-5}$ M

* Activities were calculated from the initial rate of the reaction which was linear with time for at least 15 min. K_m values shown are derived from a double reciprocal plot of 1/velocity of product formed vs. 1/substrate concentration.

† The concentration range of NADPH with 0·08 mg guinea pig microsomes equals 0·05–0·0045 mM; with 0·08 mg rat microsomes equals 0·05–0·006 mM; with 0·08 mg mouse microsomes equals 0·03–0·004 mM.

‡ The concentration range of *p*-nitroanisole with 0·8 mg guinea pig microsomes equals 0·55–0·18 mM; with 0·8 mg rat microsomes equals 1·7–0·5 mM; with 0·8 mg mouse microsomes equals 0·1–0·01 mM.

As can be observed, the K_m value of NADPH cytochrome *c* reductase for NADPH did not vary markedly in the three species; at most, a 2-fold difference was found between the mouse and guinea pig. In contrast, the K_m of *O*-demethylase for *p*-nitroanisole did vary in the three species. The K_m value for the rat was 100 times greater than the K_m value for the mouse ($1{\cdot}7 \times 10^{-3}$ compared to $1{\cdot}6 \times 10^{-5}$ M). Although the high K_m value for *p*-nitroanisole in the rat may contribute to the low *O*-demethylase activity found in this species (Table 1), the apparent affinity of *p*-nitroanisole in the guinea pig and mouse did not correlate with the *O*-demethylase activity found in these species (Table 1).

Experiments were undertaken to determine if species differences existed in the binding property of aniline and aminopyrine to microsomal cytochrome P-450,

since differences in the binding of drug substrates to this cytochrome could be an important contributory factor to species variation in the rates of drug metabolism.

Aniline–cytochrome P-450 binding spectra (type II) in various species. Aniline–cytochrome P-450 spectra obtained from mice, guinea pigs and rats are shown in Fig. 1. In each of the species examined, a typical type II spectrum was observed (trough at 390 nm; peak at 430 nm).[23] Although the shapes of the spectra were similar, the magnitude of spectral change differed. Mice had the greatest spectral change (0·023 O.D./2 mg microsomal protein), while the rat had the lowest (0·01 O.D./2 mg microsomal protein). In addition to the usual aniline type II spectrum, it was observed that upon storage of aniline-treated microsomes for at least 20 hr a shift in the type II spectrum to an atypical spectrum occurred in all species examined (Fig. 2). In contrast

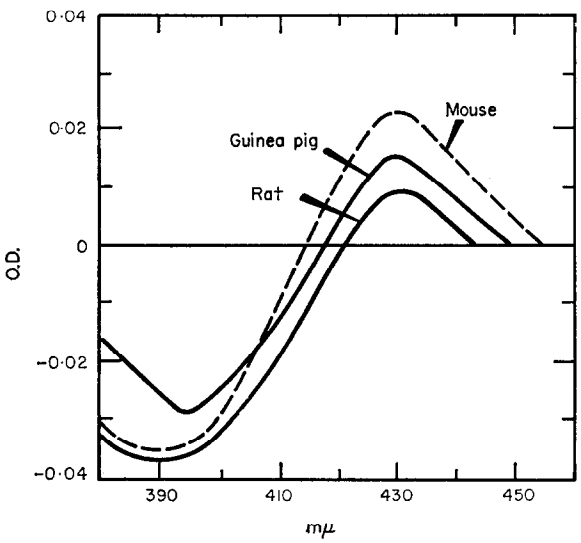

FIG. 1. Aniline (type II) substrate binding to microsomal cytochrome P-450 in various species. Difference spectra were obtained as described in Materials and Methods. The concentration of aniline was 5 mM; the concentration of microsomal protein was 2 mg/ml of 0·05 M Tris, pH 7·4. The spectra were obtained after the addition of the substrate to the microsomes.

to the similarities observed in the typical aniline type II spectra (Fig. 1), differences were found in the maximum absorption of the atypical type IIa spectra. The rat and mouse had an absorption maximum between 408–412 nm while the guinea pig had an absorption maximum between 420–425 nm. The altered spectra reflected aniline–cytochrome P-450 binding in that they could be converted to cytochrome P-450 spectra. Microsomes treated with aniline for 24 hr were reduced with sodium dithionite and complexed with CO; the appearance of cytochrome P-450 was accompanied by the disappearance of the atypical type IIa spectrum (Fig. 3). Similar results were obtained with rat or mouse microsomes treated with aniline in that the atypical aniline type IIa spectrum could be converted to the usual cytochrome P-450 spectrum.

Aminopyrine–cytochrome P-450 binding spectra (type I) in various species. In general, a typical type I spectrum (peak, 390 nm; trough 430 nm) was difficult to obtain.[23] However, storage of the microsomes with aminopyrine for at least 20 hr resulted in

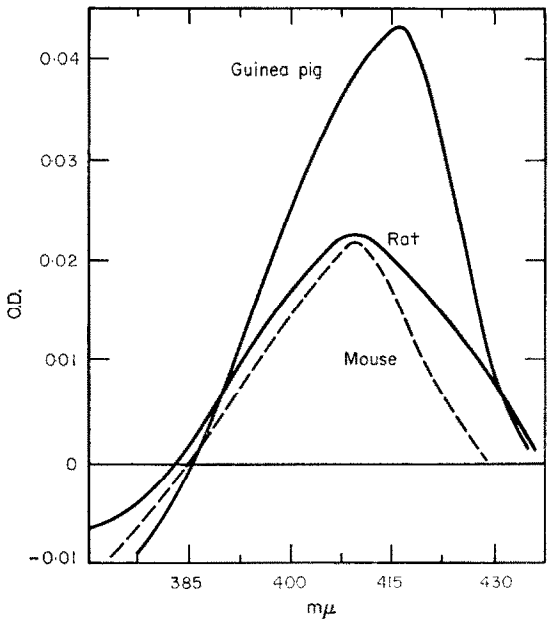

FIG. 2. Atypical aniline type IIa spectra after prolonged treatment of microsomal cytochrome P-450 with aniline in various species. Difference spectra were obtained as described in Materials and Methods. The concentrations of aniline was 5 mM; the concentration of microsomal protein was 2 mg/ml of 0·05 M Tris, pH 7·4. The spectra were obtained after the storage of the microsomes with aniline at 5° for 3 days.

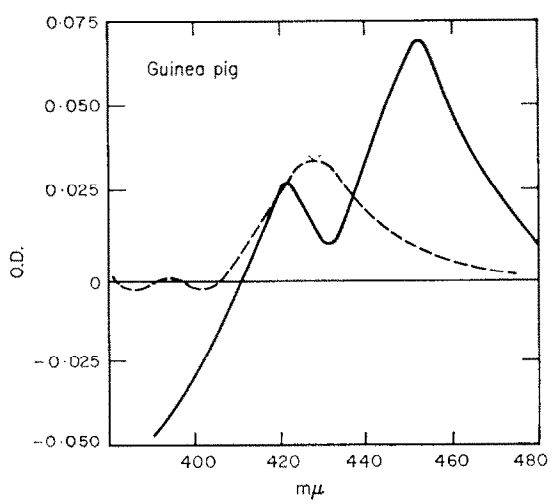

FIG. 3. Conversion of atypical aniline spectrum (type IIa) to cytochrome P-450 spectrum. Aniline atypical type IIa spectrum was obtained after the storage of guinea pig microsomes with aniline at 5° for 24 hr. The concentration of aniline was 5 mM; the concentration of microsomal protein was 2 mg/ml of 0·05 M Tris, pH 7·4. Liver microsomal cytochrome P-450 was determined by measuring the absorbance of the aniline-treated microsomes after dithionite reduction and complexing with CO, as described in Materials and Methods.

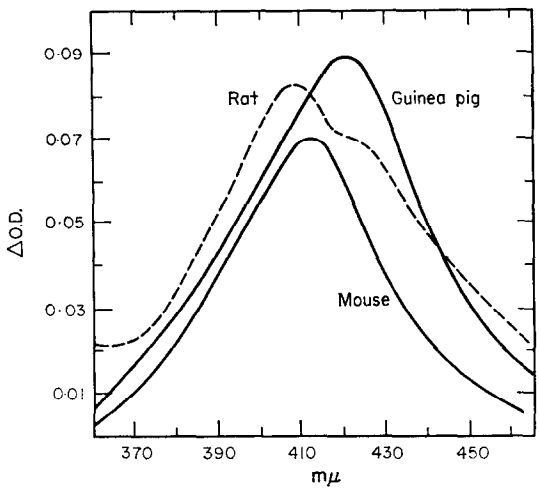

FIG. 4. Atypical aminopyrine type Ia spectra after prolonged treatment of microsomal cytochrome P-450 with aminopyrine in various species. Difference spectra were obtained as described in Materials and Methods. The concentration of aminopyrine was 5 mM; the concentration of microsomal protein was 2 mg/ml of 0·05 M Tris, pH 7·4. The spectra were obtained after 20 hr of storage of the microsomes with aminopyrine at 5°.

atypical aminopyrine–cytochrome P-450 spectra in the mouse, rat and guina pig (Fig. 4). The mouse and rat had an absorption maximum at 405–410 nm, while the guinea pig had a maximum absorption at 420 nm. As was found with atypical type IIa spectra in the guinea pig, the altered aminopyrine type Ia spectra could bo converted to cytochrome P-450 spectra (Fig. 5). Similar results were obtained with rat or mouse microsomes in that atypical aminopyrine type Ia spectra could be converted to cytochrome P-450 spectra; the conversion was accompanied by the disappearance of the type Ia aminopyrine spectrum.

In addition to converting atypical type Ia and type IIa spectra to cytochrome P-450 spectra, the quantity of cytochrome P-450 was not altered by prolonged treatment with either aminopyrine or aniline. The determination of the quantity of cytochrome P-450 in mouse and guinea pig microsomal preparations after prolonged storage (5 days) with aminopyrine or aniline is given in Table 3. In the mouse or guinea pig, the

TABLE 3. PROTECTION OF CYTOCHROME P-450 BY AMINOPYRINE OR ANILINE IN MOUSE AND GUINEA PIG LIVER MICROSOMES

		Cytochrome P-450*		
	Days of storage (5°)	No substrate treatment	Aminopyrine† treatment	Aniline† treatment
Mouse	0 (<3 hr)	0·04	0·04	0·04
	5	0·01	0·04	0·04
Guinea pig	0 (<3 hr)	0·05	0·05	0·05
	5	0·01	0·05	0·05

* Micromoles of P-450 per 100 mg of microsomal protein.

† Microsomes (2 mg/ml of 0·05 M Tris, pH 7·4) stored with aminopyrine (5 mM) or aniline (5 mM) at 5°.

quantity of cytochrome P-450 was the same, regardless of whether the microsomes were treated with aminopyrine, aniline, or not treated up to 3 hr. However, after 5 days of storage of the microsomes at 5° in the absence of aminopyrine or aniline, the cytochrome P-450 content decreased 75 per cent in mouse microsomes and 80 per cent in guinea pig microsomes. On the other hand, the quantity of cytochrome P-450 was completely protected when the microsomes were stored in the presence of these substrates in both species. Furthermore, *N*-demethylase and aniline hydroxylase activities were stable under the storage conditions; at least 80 per cent of these activities were protected when microsomes were kept with either aminopyrine or aniline at 5° from 2–5 days. The protection of aminopyrine *N*-demethylase activity by aminopyrine treatment for 2 days is shown in Table 4.

TABLE 4. PROTECTION OF AMINOPYRINE *N*-DEMETHYLASE ACTIVITY OF MOUSE, GUINEA PIG AND RAT MICROSOMES BY AMINOPYRINE

		Aminopyrine *N*-demethylase activity*	
	Days of storage (5°)	No aminopyrine treatment	Aminopyrine† treatment
Mouse	0	22·9	22·9
	2	4·0	15·2
Guinea pig	0	4·6	4·6
	2	2·6	3·5
Rat	0	11·0	11·0
	2	1·9	7·8

* Enzyme activity = micromoles of product formed per hr per 100 mg of microsomal protein at 27°.

† Microsomes (2 mg/ml of 0·05 M Tris, pH 7·4) stored with aminopyrine (5 mM) at 5°.

The possibility was also examined that specific electron transport components influenced the rate of a particular type of drug oxidation reaction (*O*-demethylation, *N*-demethylation or ring hydroxylation). This problem was investigated with the use of drug enzyme inducers such as phenobarbital and 3-methylcholanthrene since these agents can cause an increase in the quantity of specific microsomal electron transport components. Time course studies were carried out to observe if an increase in a specific component resulted in an increase in a particular type of drug oxidation activity.

Induction studies. Swiss–Webster, male mice (10–15 g) were given phenobarbital (50 mg/kg) two times each day. Groups of six mice were sacrificed after the first day of phenobarbital treatment, other groups after the second, third and fourth day. Cytochrome P-450 content, NADPH cytochrome P-450 reductase, *O*-demethylase, *N*-demethylase and aniline hydroxylase activities were determined in phenobarbital- and non-phenobarbital-treated mice. As can be observed in Fig. 6, the quantity of cytochrome P-450 reached its highest level after 2 days, remaining at that level on the third and fourth day. *N*-demethylase and *O*-demethylase activities were also at their highest level on the third and fourth day. In contrast, NADPH cytochrome P-450 reductase required 2·5 days to reach its maximum activity and dropped almost 4-fold

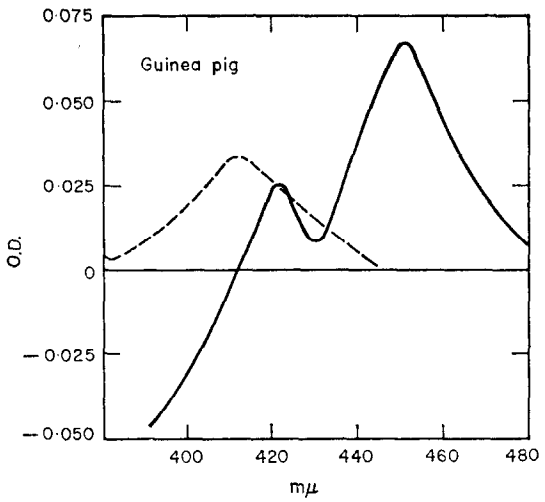

FIG. 5. Conversion of atypical aminopyrine (type Ia) spectrum to cytochrome P-450 spectrum. Aminopyrine type Ia spectrum was obtained after storage of guinea pig microsomes with aminopyrine at 5° for 24 hr as described in Materials and Methods. The concentration of aminopyrine was 5 mM; the concentration of microsomal protein was 2 mg/ml of 0·05 M Tris, pH 7·4. Liver microsomal cytochrome P-450 was determined by measuring the absorbance of the aminopyrine-treated microsomes after dithionite reduction and complexing with CO, as described in Materials and Methods.

by the fourth day. Its time course of induction and decline correlated well with aniline hydroxylase activity (Fig. 7). A difference also existed in the time sequence of induction between NADPH cytochrome *c* reductase activity and NADPH cytochrome P-450 reductase activity. NADPH cytochrome *c* reductase activity showed an increase in activity on each of the 4 days while NADPH cytochrome P-450 reductase activity had a peak value between the second and third day followed by a decrease on the fourth day (Table 5). These results would indicate that the reductase activity responsible for the reduction of cytochrome P-450 is not identical with that responsible for the reduction of cytochrome *c*.[19,25,26]

TABLE 5. NADPH CYTOCHROME P-450 REDUCTASE AND NADPH CYTOCHROME *c* REDUCTASE ACTIVITY AT VARIOUS TIMES AFTER PHENOBARBITAL TREATMENT

Activity*	Phenobarbital† (days of treatment)				
	No PB	1	2	3	4
NADPH cytochrome *c* reductase	168 ± 8·5	235 ± 14	346 ± 18	370 ± 20	444 ± 24
NADPH cytochrome P-450 reductase	8·6 ± 1·1	14 ± 1·0	42 ± 2·4	32 ± 1·7	18 ± 1·2

* Enzyme activity = micromoles of product formed per hour per 100 mg of microsomal protein at 27°.

† Groups of twelve Swiss–Webster, male mice (10–15 g) were injected, i.p., with phenobarbital (50 mg/kg) twice each day for the days given in the Table. The activity of NADPH cytochrome *c* reductase and NADPH cytochrome P-450 reductase was determined on six liver preparations; each preparation of microsomes was prepared from two livers. Mean ± S.E. of six determinations.

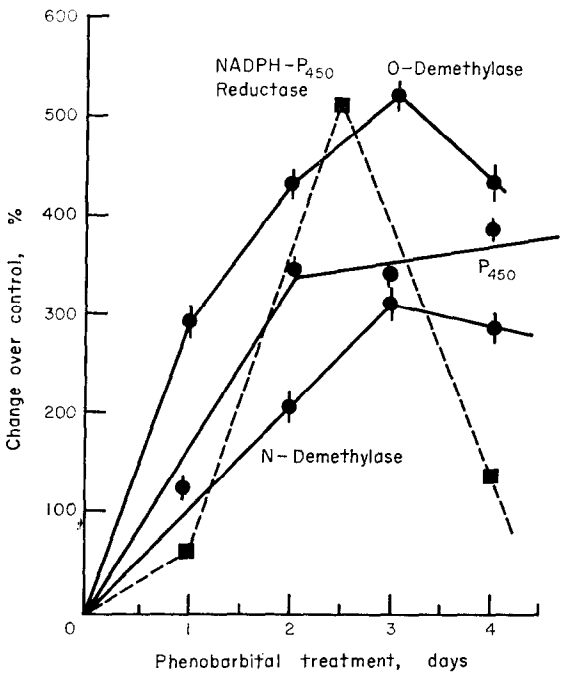

FIG. 6. Cytochrome P-450, NADPH cytochrome P-450 reductase activity, aminopyrine *N*-demethylase and *p*-nitroanisole *O*-demethylase activities in mice at various times after phenobarbital administration. Groups of twelve Swiss–Webster, male mice (10–15 g) were injected, i.p., with phenobarbital (50 mg/kg) two times each day for the days given in the figure. The quantity of cytochrome P-450 and enzyme activities were determined on six liver preparations; each preparation of microsomes was prepared from two livers.

Results are expressed as per cent increase over control level. Control levels: cytochrome P-450 = 0·6 ± 0·02 μmoles/g microsomal protein; NADPH cytochrome P-450 reductase activity = 86 ± 11 μmoles product formed/hr/g microsomal protein; aminopyrine *N*-demethylase activity = 140 ± 8·3 μmoles product formed/hr/g microsomal protein; *p*-nitroanisole *O*-demethylase activity = 40 μmoles ± 3·3 product formed/hr/g microsomal protein.

The use of 3-methylcholanthrene as an inducer gave similar results to those found with phenobarbital in that the rate of *O*-demethylation was dependent upon the quantity of cytochrome P-450 and the activity of NADPH cytochrome P-450 reductase determined the rate of aniline hydroxylation (Table 6). The quantity of cytochrome P-450 and *O*-demethylase activity reached their highest level 24 hr after 3-methylcholanthrene administration, maintaining that level up to 42 hr. In contrast, aniline hydroxylase activity was dependent upon the activity of NADPH cytochrome P-450 reductase. The reductase activity had a peak level at 24 hr as did aniline hydroxylase activity, and both activities declined to control levels by 42 hr. As was found with phenobarbital, 3-methylcholanthrene induction resulted in differences in the time sequence of induction of NADPH cytochrome *c* reductase and NADPH cytochrome P-450 reductase activities; in 42 hr NADPH cytochrome P-450 reductase activity had returned to control levels while NADPH cytochrome *c* reductase activity showed a 90 per cent increase over control levels.

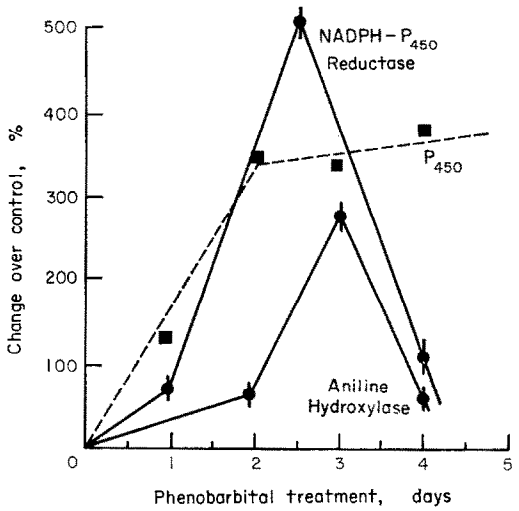

FIG. 7. NADPH cytochrome P-450 reductase activity, cytochrome P-450 and aniline hydroxylase activity in mice at various times after phenobarbital administration. Groups of twelve Swiss–Webster, male mice (10–15 g) were injected, i.p., with phenobarbital (50 mg/kg) two times each day for the days given in the figure. The quantity of cytochrome P-450 and enzyme activities were determined on six liver preparations; each preparation of microsomes was prepared from two livers.

Results are expressed as per cent increase over control level. Control levels: cytochrome P-450. = 0·6 ± 0·02 μmoles/g microsomal protein; NADPH cytochrome P-450 reductase = 86 ± 11 μmoles product formed/hr/g microsomal protein; aniline hydroxylase activity = 1·4 ± 0·1 μmoles product formed/hr/g 15,000 *g* supernatant fraction protein.

TABLE 6. ACTIVITY OF DRUG ENZYMES AND MICROSOMAL ELECTRON TRANSPORT COMPONENTS IN MICE AT VARIOUS TIMES AFTER 3-METHYLCHOLANTHRENE ADMINISTRATION*

Activity†	No 3-MC	With 3-MC		
	(0 Time)	12 hr	24 hr	42 hr
Cytochrome P-450	0·06	0·07	0·10	0·11
p-NO_2 anisole *O*-demethylase	1·9	1·7	3·8	3·4
NADPH cytochrome P-450 reductase	8·6	8·0	42·0	8·0
Aniline hydroxylase‡	0·14	0·08	0·27	0·12
NADPH cytochrome *c* reductase	168	250	322	307

* Assay conditions and 3-methylcholanthrene treatment (80 mg/kg, once a day) are given in Materials and Methods.

† Cytochrome P-450 = micromoles per 100 mg microsomal protein; *p*-NO_2 anisole *O*-demethylase, NADPH cytochrome P-450 reductase and NADPH cytochrome *c* reductase = micromoles of product formed per hour per 100 mg of microsomal protein at 27°.

‡ Aniline hydroxylase = micromoles of product formed per hr per 100 mg of 15,000 *g* supernatant protein.

DISCUSSION

The studies reported on typical drug oxidation reactions are in keeping with previous studies in that there is no obvious correlation in the microsomal oxidation of such substrates as aminopyrine, aniline, or *p*-nitroanisole in a number of species, i.e. the

species which exhibited the highest rate of *p*-nitroanisole *O*-demethylase activity, did not necessarily have the highest rate of aminopyrine *N*-demethylase or aniline hydroxylase activity.[26] Investigation of the quantity and activity of liver microsomal electron transport components in various species did, however, allow the elimination of certain electron transport components as important rate determinants and enabled a more critical evaluation of those electron transport components which play a role in drug oxidation. For example, the quantity of microsomal cytochrome b_5 and NADPH cytochrome *c* reductase activity, as well as the apparent affinity of the microsomal system for NADPH or drug substrate, such as *p*-nitroanisole, could not, in general, account for the observed species variation (Tables 1 and 2). Recent evidence by Estabrook *et al.*[27] has indicated that cytochrome b_5 may act as the donor of an electron to oxygenated P-450. Although the precise role of cytochrome b_5 in drug hydroxylation must still be determined,[28–31] the present studies indicate that it is not a critical factor for overall drug oxidation. The level of NADPH cytochrome *c* reductase activity also does not appear to be an important rate determinant of drug oxidation. The difference in NADPH cytochrome *c* reductase activity was only 35 per cent in the mouse, rat and guinea pig, yet overall drug oxidation as determined by *O*-demethylase, *N*-demethylase and aniline hydroxylase activities in these species varied up to 3-fold (Table 1).

A greater correlation exists between overall drug oxidation and the quantity of cytochrome P-450 reductase activity and cytochrome P-450 in various species. For example, the level of cytochrome P-450 reductase activity in the guinea pig and rat (36 μmoles/hr/g microsomal protein compared to 10 μmoles) correlated with the *O*-demethylase activity in these species (70 μmoles/hr/g microsomal protein compared to 20 μmoles, Table 1). NADPH cytochrome P-450 reductase activity in the mouse and guinea pig (86 μmoles/hr/g microsomal protein compared to 36 μmoles) could also account for their relative *N*-demethylase activity (140 μmoles/hr/g microsomal protein compared to 70 μmoles, Table 1). However, the quantity of reductase activity in the rat could not explain the high *N*-demethylase activity in this species. Differences in the quantity of cytochrome P-450 can also account for some of the species variation found in overall drug oxidation. The higher quantity of cytochrome P-450 in the rat compared to the guinea pig (1·0 μmoles/g microsomal protein compared to 0·7 μmole) can account for the higher *N*-demethylase and aniline hydroxylase activities in this species (Table 1). Although the quantity of cytochrome P-450 and NADPH cytochrome P-450 reductase activity were, in general, the most important factors involved in determining drug oxidation activity, they could not account for all the observed species variation.

The drug substrate–cytochrome P-450 binding studies indicate that this initial step in drug oxidation may play an important role in species differences. The general importance of drug binding to cytochrome P-450 has been known for some time.[23,32–35] In fact, recently it has been suggested that substrate–cytochrome P-450 spectral changes may be visible representations of Michaelis–Menten complexes.[36] Of particular interest in the present studies were the differences in the atypical substrate binding spectra obtained after prolonged treatment of microsomes with either a type I substrate (aminopyrine) or type II substrate (aniline) (Figs. 2 and 4). The atypical spectra may reflect a qualitative difference in the form of cytochrome P-450 in various species and explain the inability to account for variation in drug oxidation solely

by the quantity of cytochrome P-450. Previously, qualitative differences in cytochrome P-450 were not apparent since a comparison was generally made between the reduced cytochrome P-450-CO complex or usual type I and type II substrate binding spectra in various species. Atypical type Ia spectra (aminopyrine) and atypical type IIa spectra (aniline) of guinea pig microsomes had a maximum absorption at longer wavelengths (420 nm) compared to mouse or rat microsomes (405–410 nm). In addition, it was possible to convert the atypical spectra to the usual cytochrome P-450-CO spectrum, and this conversion was accompanied by the disappearance of the atypical spectra. Furthermore, the cytochrome P-450 present after prolonged treatment with substrate was active and able to function in the oxidation of drugs. Of interest, in light of these spectral differences, is the observation that the guinea pig had a lower rate of metabolism of both aminopyrine and aniline than the mouse or rat. Our studies are in keeping with those of Alvares *et al.*[37] who found species differences in the oxidized spectra of microsomal cytochrome P-450 induced by phenobarbital. These investigators reported that the spectrum of oxidized cytochrome P-450 of mice and rats had a maximum absorption at 418 nm while oxidized cytochrome P-450 of the guinea pig had a maximum absorption at 421 nm.

It is also apparent that the type of hydroxylation reaction studied (*N*-demethylation, *O*-demethylation or aniline hydroxylation) is an important consideration in determining the role of particular electron transport components in overall drug oxidation. Our studies employing phenobarbital or 3-methylcholanthrene to induce individual electron transport components have indicated that *N*-demethylase and *O*-demethylase activities are dependent upon the quantity of cytochrome P-450 and that aniline hydroxylase activity is dependent on the activity of NADPH cytochrome P-450 reductase. Furthermore, the time course of induction of cytochrome P-450 correlated with the time course of induction of *O*-demethylase and *N*-demethylase activities while the pattern of induction and decline of aniline hydroxylase activity was dependent on NADPH cytochrome P-450 reductase activity. In this regard, perhaps animal data would be more meaningful in its extrapolation to man if both the quantity of individual electron transport components and the type of drug oxidation reaction under study are considered.

REFERENCES

1. J. J. Burns, M. Weiner, G. Simson and B. B. Brodie. *J. Pharmac. exp. Ther.* **108,** 33 (1953).
2. J. Axelrod, *J. Pharmac. exp. Ther.* **110,** 315 (1954).
3. J. J. Burns, B. L. Berger, P. A. Lief, A. Wollack, E. M. Papper and B. B. Brodie, *J. Pharmac. exp. Ther.* **114,** 289 (1955).
4. G. J. Dutton and C. G. Greig, *Biochem. J.* **66,** 52p (1957).
5. G. P. Quinn, J. Axelrod and B. B. Brodie, *Biochem. Pharmac.* **1,** 152 (1958)
6. H. G. Bray, T. J. Franklin and S. P. James, *Biochem. J.* **73,** 465 (1959).
7. D. V. Parke, *Biochem. J.* **77,** 493 (1960).
8. J. V. Dingell, F. Sulser, and J. R. Gillette, *Fedn. Proc.* **21,** 184a (1962).
9. W. A. M. Duncan, *Proc. Eur. Soc. Drug Toxicity* **2,** 67 (1963).
10. J. R. Gillette, *Ann. N.Y. Acad. Sci.* **123,** 42 (1965).
11. J. J. Burns, in *Evaluation of New Drugs in Man* (Ed. E. Zaimis), p. 21. Pergamon, Oxford (1965).
12. D. S. Davies, P. L. Gigon and J. R. Gilette, *Life Sci.* **8,** 85 (1969).
13. R. T. Williams, in *Fundamentals of drug metabolism and drug disposition* (Eds. B. N. La Du, H. G. Mandel and E. L. Way) p. 187. Williams & Wilkins, Baltimore (1971).
14. K. J. Netter and G. Seidel, *J. Pharmac. exp. Ther.* **146,** 61 (1964).

15. V. G. ZANNONI, in *Fundamentals of drug metabolism and drug disposition* (Eds. B. N. LA DU, H. G. MANDEL, and E. L. WAY) p. 566. Williams & Wilkins, Baltimore (1971).
16. T. NASH, *Biochem. J.* **55,** 416 (1953).
17. B. B. BRODIE and J. AXELROD, *J. Pharmac. exp. Ther.* **94,** 22 (1948).
18. C. H. WILLIAMS and H. J. KAMIN, *J. biol. Chem.* **237,** 587 (1962).
19. J. L. HOLTZMAN, T. E. GRAM, P. L. PIGEON and J. R. GILLETTE, *Biochem. J.* **110,** 507 (1968).
20. V. G. ZANNONI, E. J. FLYNN and M. LYNCH, *Biochem. Pharmac.* **21,** 1377 (1972).
21. T. OMURA and R. SATO, *J. biol. Chem.* **239,** 2370 (1964).
22. T. OMURA and R. SATO, *J. biol. Chem.* **239,** 2379 (1964).
23. H. REMMER, J. SCHENKMAN, R. W. ESTABROOK, H. SASAME, J. R. GILLETTE, S. NARASIMHULU, D. Y. COOPER and O. ROSENTHAL, *Molec. Pharmac.* **2,** 187 (1966).
24. O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
25. R. SATO, T. OMURA and H. NISHIBAYASCHI, in *Oxidases and related redox system* (Eds. T. E. KING, H. S. MASON and M. MORRISON), Vol. 2, p. 861. John Wiley, New York (1965).
26. H. B. HUCKER, *A. Rev. Pharmac.* **10,** 99 (1970).
27. R. W. ESTABROOK, A. G. HILDEBRANDT, J. BARON, K. J. NETTER and K. LEIBMAN, *Biochem. biophys. Res. Commun.* **42,** 132 (1971).
28. C. F. STRITTMATTER and E. G. BALL, *Proc. natn. Acad. Sci. U.S.A.* **38,** 191 (1952).
29. H. S. MASON, J. C. NORTH and M. VANNESTE, *Fedn Proc.* **24,** 1172 (1965)
30. D. GARFINKLE, *Archs Biochem. Biophys.* **71,** 111 (1957).
31. P. STRITTMATTER and S. F. VELICK, *J. biol. Chem.* **228,** 785 (1957).
32. Y. IMAI and R. SATO, *Biochem. biophys. Res. Commun.* **22,** 620 (1966).
33. J. B. SCHENKMAN, H. REMMER and R. W. ESTABROOK, *Molec. Pharmac.* **3,** 113 (1967).
34. P. L. GIGON, T. E. GRAM and J. R. GILLETTE, *Molec. Pharmac.* **5,** 109 (1969).
35. G. J. MANNERING, in *Fundamentals of drug metabolism and drug disposition* (Ed. B. N. LA DU, H. G. MANDEL and E. L. WAY) p. 206. Williams & Wilkins, Baltimore (1971).
36. J. R. GILLETTE, *Biochemical aspects of antimetabolites and of drug hydroxylation,* Vol. 16 in the Federation of European Biochemical Society Symposia Series (Ed. D. SHUGAR) p. 109. Academic Press, New York (1969).
37. A. P. ALVARES, G. SCHILLING and W. LEVIN, *J. Pharmac. exp. Ther.* **175,** 4 (1970).