CYTOCHROME P-450 AND ITS ROLE IN DRUG METABOLISM[1]

6528

JAMES R. GILLETTE, DONALD C. DAVIS, AND HENRY A. SASAME
Laboratory of Chemical Pharmacology, National Heart and Lung Institute, Bethesda, Maryland

It is now almost universally recognized that most drugs undergo a variety of metabolic changes before they are excreted in the urine, bile, or air. Since drugs have widely different structures, it is not surprising that virtually every enzyme in the body may be considered as a drug-metabolizing enzyme. Indeed many drugs are specifically designed to resemble substances normally synthesized and catabolized in the body and thus are metabolized by specific enzymes, which synthesize and catabolize substances such as catecholamines, indoles, nucleic acids, amino acids, steroids, and fats. Most drugs, however, either are metabolized slowly by these specific enzymes or have no endogenous counterpart and thus are metabolized by nonspecific enzymes.

In recent years, many investigators have focused their attention on the metabolism of drugs by a group of nonspecific enzymes in liver endoplasmic reticulum. The versatility of these enzymes is unique in biochemistry for they catalyze such widely diverse reactions as the oxidation of alkanes and aromatic compounds, the epoxidation of alkenes, polycyclic hydrocarbons, and halogenated benzenes, the dealkylation of the secondary and tertiary amines, the conversion of amines to N-oxide, hydroxylamine, and nitroso derivatives, the oxidative cleavage of ethers and organic thiophosphate esters, and the conversion of phosphothionates to their phosphate derivatives. In addition, these enzymes may catalyze the reduction of azo-compounds and nitro-compounds to primary aromatic amines and possibly the reductive cleavage of halogenated alkanes, such as carbon tetrachloride, to free radicals (1–4).

Since the oxidative reactions in the hepatic endoplasmic reticulum require both NADPH (or NADH) and oxygen, they are frequently called mixed-function oxidases, according to the nomenclature of Mason (5), or

[1] Abbreviations used in this chapter are: DPEA: 2,4-dichloro-6-phenylphenoxyethylamine; ESR: electron spin resonance; FAD: flavin adenine dinucleotide; Lilly 18947: 2,4-dichloro-6-phenylphenoxyethyl diethylamine; MEOS: microsomal ethanol oxidizing system; NADP: nicotinamide adenine dinucleotide phosphate; NADPD: deuteriated nicotinamide adenine dinucleotide phosphate; NADPH: reduced nicotinamide adenine dinucleotide phosphate.

monooxygenases according to the nomenclature of Hayaishi (6). But whether the reactions are catalyzed by a single enzyme or a family of different enzyme systems in hepatic endoplasmic reticulum is still being investigated. In this regard, it is noteworthy that a metabolite may be formed in the body by way of different pathways or by a number of enzyme systems, the relative importance of which may vary not only with the drug substrate but also with the animal species or with different treatments.

In this review, we shall discuss recent studies with hepatic microsomal enzymes that have helped to elucidate the pathways of drug metabolism, the enzyme systems that catalyze the oxidative and reductive reactions, and the mechanisms of the enzyme systems. We shall also discuss interrelationships between the oxidative enzymes in the endoplasmic reticulum and other enzymes involved in drug metabolism in other organelles of liver.

Mechanisms of NADPH-Dependent Oxidative Enzyme Systems in Liver Microsomes

In most of the oxidative reactions catalyzed by liver microsomes, the substrate first combines with the oxidized form of a carbon monoxide sensitive hemoprotein called cytochrome P-450. The substrate-cytochrome P-450 complex then is reduced by an electron from the flavoprotein, NADPH-cytochrome *c* reductase, to form a reduced substrate-cytochrome P-450 complex which in turn reacts with oxygen to form a substrate-cytochrome P-450 oxygen complex. Although the sequence of events after this stage is still uncertain, it is believed that a second electron reduces the substrate-cytochrome P-450-O_2 complex to form an active oxygen intermediate that decomposes with the formation of the product and the oxidized cytochrome P-450. Since liver microsomes contain considerably more cytochrome P-450 than FAD, it has become obvious that a molecule of NADPH-cytochrome *c* reductase must reduce a number of molecules of cytochrome P-450 (3, 4).

Spectral changes of cytochrome P-450 from liver microsomes.—Various substrates and inhibitors combine with the oxidized form of cytochrome P-450 and thereby cause small but significant changes in its absorbance spectrum (7–9). In general two types of changes are observed in the difference spectrum, i.e. the absorbance spectrum of liver microsomes in the presence of substrate or inhibitor (experimental cuvette) minus the spectrum of liver microsomes in the absence of substrate (reference cuvette). Some substances, such as hexobarbital, aminopyrine, and ethylmorphine, cause a minimum in the difference spectrum at 420 nm and a maximum in the difference spectrum at about 390 nm; compounds that cause this kind of spectral change have been called type I compounds. Other substances, such as nicotinamide, aniline, DPEA (2,4-dichloro-6-phenylphenoxyethylamine) (7–9), and octylamine (10) cause a maximum in the difference spectrum at about 430 nm and a minimum in the difference spectrum at about 390

to 410 nm; compounds that cause this type of change have been called type II substances.

Cytochrome P-450 reduction by NADPH.—Gigon et al (11) found that in a CO atmosphere type I substances accelerate the reduction of cytochrome P-450, whereas type II substances decelerate it. Moreover, NADPH-oxidation in air was stimulated by type I substances, but was either unaffected or inhibited by type II substances.

Although Gigon et al (11) used the initial rates of cytochrome P-450 reduction in calculating the relationships between drug metabolism and cytochrome reduction, semilogarithmic plots of the data showed that the rate of cytochrome P-450 reduction was polyphasic and not first order. The slower phases seemed to be unusually sensitive to oxygen since addition of electron transport particles and succinate, which scavenge traces of oxygen present after reducing and gassing the system with CO, markedly increased the reduction rate of the slower phases. Subsequently, Sasame showed that the effect of low concentrations of oxygen on the slower phases of cytochrome P-450 reduction was due to an unusually slow reduction rather than to an unusually rapid oxidation of cytochrome P-450 (see Gillette, 12).

Diehl et al (13) suggested that the rapidly reduced cytochrome P-450 followed first order kinetics and the slowly reduced followed second order kinetics. However, Sasame[2] found that plots of the logarithm of the rate of cytochrome P-450 reduction against the logarithm of the amount of unreduced cytochrome P-450 were linear with a slope of about 2.4. These findings are thus inconsistent with the view that the order of the reaction changes with time. Instead they suggest that cytochrome P-450 exists in several pools. These pools of cytochrome P-450 could represent differences in the relative amounts of cytochrome P-450 and NADPH-cytochrome *c* reductase in the population of microsomes. On the other hand, these pools may represent different forms of cytochrome P-450 that inherently are reduced at different rates.

Cytochrome b_5 and NADH in drug metabolism.—The requirement of cytochrome P-450 enzymes for NADPH can be partially replaced by NADH, but the activities are invariably lower. Although it has been generally assumed that the electrons from NADH entered the system by way of NADH-cytochrome b_5 reductase or by another NADH-dependent flavoprotein, Omura[3] found that the antibody to NADPH-cytochrome *c* reductase blocked the hydroxylation of aniline and the demethylation of aminopyrine when either NADH or NADPH were present, but that the antibody to NADH-cytochrome b_5 reductase blocked neither the NADH nor the NADPH mediated reaction. Thus NADH must act through NADPH-cyto-

[2] Sasame, H. A. and Gillette, J. R., unpublished observations.

[3] Omura, T., personal communication.

chrome *c* reductase when it is used as the cofactor for cytochrome P-450 enzymes.

Despite these findings of Omura[3], many investigators have found that NADH in the presence of saturating concentrations of NADPH increases the activity of the cytochrome P-450 enzyme systems, but the reason for the stimulation by NADH has been unclear. During long incubation periods NADH might protect NADPH from inactivation by NADPH pyrophosphatase (3). Alternatively, NADH-dependent flavoproteins might compete with NADPH-cytochrome *c* reductase for the reduction of endogenous substrates, such as cytochrome b_5. In accord with the latter view, Correia & Mannering (14) found that stearoyl-CoA inhibited ethylmorphine N-demethylation and that cyanide not only prevented the inhibition but actually enhanced the activity, presumably by blocking the cytochrome b_5 mediated pathway of stearoyl-CoA desaturase. Presumably, p-cresol and a number of other phenols may also inhibit drug metabolism by this mechanism, since Oshino & Sato (15) found that these substances also stimulate cytochrome b_5 oxidation, especially in liver microsomes from rats receiving a high carbohydrate diet.

Preventing the passage of electrons from NADPH through the cyanide-sensitive fatty acid desaturase, however, cannot be the only way that NADH enhances the metabolism of drugs. Hildebrandt & Estabrook (16) found that NADH-oxidation was stimulated only slightly by substrates, such as aminopyrine, but that NADH-oxidation was greatly stimulated when the system contained both the substrate and saturating concentrations of a NADPH-generating system. Since NADPH was kept fully reduced by the generating system, the effect could not be attributed to a transhydrogenation reaction. Because high concentrations of NADPH were used, NADH could not be acting by keeping NADPH-cytochrome *c* reductase more fully reduced nor by enhancing the rate of cytochrome P-450-substrate reduction. Since the NADPH-generating system was required for the augmented NADH-oxidation, the increase in NADH-oxidation could not represent a NADH-dependent, substrate-stimulated, electron transport system. These considerations thus led Hildebrandt & Estabrook (16) to suggest that NADPH preferentially donates an electron for the reduction of the cytochrome P-450-substrate and that either NADPH or NADH could donate the second electron required by the mixed function oxidase mechanism.

Spectrophotometric studies caused them to suggest that cytochrome b_5 mediates the transfer of the second electron into the cytochrome P-450 system. They observed that in air, but in the absence of substrate, cytochrome b_5 was fully reduced by NADPH, but addition of a substrate, such as aminopyrine, caused a decrease in the absorbancy in that part of the difference spectrum usually attributed to reduced cytochrome b_5. Subsequent addition of NADH reversed the decrease in absorbancy.

Estabrook's group also observed that in air and in the presence of NADPH, the addition of a substrate, such as hexobarbital, ethylmorphine,

MECHANISM OF CYTOCHROME P–450 ENZYME SYSTEMS

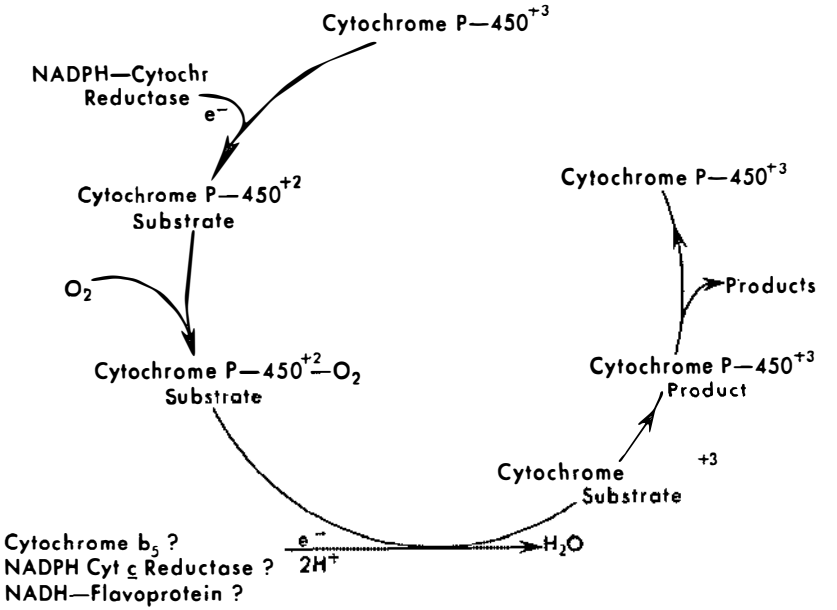

FIG. 1

or aminopyrine, caused the formation of a peak at about 440 nm of the difference spectrum (17). Since this peak disappeared when the system became anaerobic, they suggested that it was due to the oxygenated cytochrome P-450-substrate complex. Moreover, NADH prevented the formation of the peak, suggesting that the second electron donated by cytochrome b_5 entered the cytochrome P-450 system after the oxygenation step.

The schema presented in Figure 1 summarizes the current view of Estabrook's group. Attractive though this concept of the cytochrome P-450 mechanism may seem, there are a number of inconsistencies that must be resolved before it can be completely accepted: (*a*) Omura[3] has found that antibodies against cytochrome b_5 do not inhibit the metabolism of either aniline or aminopyrine. (*b*) In the presence of substrate the rate of cytochrome b_5 reduction by NADPH in rat liver microsomes is at least an order of magnitude faster than either the rate of cytochrome P-450 reduction in CO, or the rate of NADPH-oxidation in air.[2] It is therefore difficult to conceive how reduction of cytochrome b_5 could be a rate-controlling step in drug metabolism, as is implied by the spectral studies of Estabrook. (*c*) Although we (18) have confirmed the findings of Hildebrandt & Estabrook (16) that NADH stimulates the metabolism of aminopyrine and ethylmorphine and

slows the total oxidation of NADPH, we also found that NADH slows the oxidation of NADPH in $CO:O_2$ atmosphere of 9:1. As a result, the ratio of "CO-inhibitable" NADPH-oxidation to "CO-inhibitable" drug metabolism was not decreased by NADH as would be expected if NADH were supplying the second electron required for the metabolism of the drugs. For these reasons the mechanism of the drug-metabolizing enzymes suggested by Estabrook's group remains a plausible but thus far an unproved concept.

Cytochrome P-450 of Pseudomonas putida.—Owing to the difficulties involved in studying the mechanism of cytochrome P-450 enzymes in impure particulate preparations, most of the currently accepted knowledge concerning the properties of cytochrome P-450 comes by analogy from purified preparations of the soluble cytochrome P-450 from *P. putida* (19, 20).

The cytochrome P-45O system from *P. putida* catalyzes the hydroxylation of D-camphor and certain camphor analogs but does not catalyze the hydroxylation of steroids, hexobarbital, or laurate. Owing to its substrate specificity this cytochrome system has frequently been called P-450 cam. Like the 11-β hydroxylase system of adrenal mitochondria (21), the P-450 cam system is reduced by a nonheme iron protein, but unlike the adrenal system the *P. putida* nonheme iron protein, called putidaredoxin, is reduced by a NADH-dependent flavoprotein (22) rather than a NADPH-dependent flavoprotein (21).

Studies (20, 21) of the spectral characteristics of P-450 cam showed

TABLE 1—ABSORPTION SPECTRA OF P-450 CAM[a]

	−Substrate		+Substrate	
	λ nm	ε mM	λ nm	ε mM
Oxidized	417–8	104–5	391–2	87–101
	535	10–10.3	540	10
	570–1	10.4–10.5	643–6	4.5–5.0
Reduced	408–11	69–71	408–9	73–83
	540	13.5–14	540–5	14–15.2
Oxygenated			418	62
			552	14
CO-complex	447	104	446–7	106–19
	550	12.1	550–2	12–13
CO difference spectrum			446 minus 490	95

[a] Composite from Peterson, J. A. (20) and Gunsalus, I. C. (personal communication).

that the oxidized form has a peak at 417–8 nm (Table 1). On the addition of D-camphor to P-450 cam the peak at 417–8 nm disappears and a new peak appears at 391–2 nm. This spectral change is thus analogous to the type I spectral change observed in liver microsomes with substrates, such as ethylmorphine, aminopyrine, and hexobarbital. By contrast, on addition of aniline or 1-phenylimidazole to the oxidized form of P-450 cam, the peak at 417–8 nm is shifted to about 423 nm without any appreciable change in intensity. This spectral change is thus analogous to the type II spectral change observed on the addition of pyridine derivatives, such as nicotinamide, and primary amines, such as aniline, DPEA, and octylamine, to liver microsomes. One of the most important observations, however, was the finding that the addition of 1-phenylimidazole to P-450 cam in the presence of D-camphor causes the peak at 391–2 nm to disappear and a peak at 423 to appear, indicating that a type II substance displaces a type I substance from oxidized P-450 cam.

At 423 nm the absorbancy of the camphor-P-450 cam complex is less than that of the free form of P-450 cam. For this reason, the difference spectrum caused by a type II substance will appear to be greater when D-camphor is present in both the experimental and reference cuvettes than when it is absent from both cuvettes. However, it is not certain that all type II substances would produce exactly the same spectral change on combination with oxidized P-450 cam, because addition of ethylisocyanide to oxidized P-450 cam in the presence of D-camphor causes the formation of a peak at about 430 nm rather than at 423 nm (23). Interpretation of difference spectra with other type II substances may, therefore, be extraordinarily difficult.

When cyclohexanone is added to oxidized P-450 cam, the peak at 391–2 nm disappears and a peak at 418 nm appears (24). This change thus represents a displacement of a type I substance from oxidized P-450 cam and is analogous to the "atypical type II" spectral change obtained with cortisol (9) or the displacement of endogenous substances by butanol (13).

After reduction with dithionite the free form of P-450 cam has an absorbance peak at 411 nm whereas the P-450 cam substrate complex has a peak at 408 nm. Titration studies revealed that under anaerobic conditions only one electron from either reduced putidaredoxin or dithionite was required to reduce the P-450 cam substrate complex. After the substrate complex was stoichiometrically reduced, addition of CO caused the formation of a peak at 447 nm (which represents the formation of P-450 cam-CO-substrate complex) whereas introduction of oxygen causes the formation of a peak at 418 nm (which presumably represents the P-450-cam-O_2 substrate complex) (25). Although the oxygenated complex is rather stable when stoichiometric amounts of putidaredoxin are used ($t\text{-}\frac{1}{2} = 5$ min at room temperature and 40 nm at 6°), it rapidly decomposes after addition of an excess of reduced putidaredoxin. These findings indicate that the second electron required by the mixed function oxidase mechanism enters the system after the formation of the oxygenated P-450 cam substrate complex.

Electron spin resonance (ESR) studies of the various forms of P-450 cam have been useful in elucidating the sequence of events and the properties of cytochrome P-450 in liver microsomes (20). The iron in the free form of oxidized P-450 cam is low spin (one unpaired electron) as shown by absorption bands at $g = 1.91$, 2.26, and 2.44. Addition of D-camphor converts the oxidized P-450 cam to a high spin form (five unpaired electrons) as shown by absorption bands at $g = 8$, 4, and 1.98 which are obtained at liquid helium temperatures (15° K) but not at room temperature. Subsequent addition of 1-phenylimidazole converts oxidized P-450 cam back to a low spin form. Addition of reduced putidaredoxin to the oxidized P-450 cam-camphor complex also converts the iron from a high spin Fe^{+3} form to a low spin Fe^{+2} form. These findings are consistent with the view of Peterson (20) that the camphor displaces from the iron of oxidized P-450 cam a strong field ligand, such as cysteine sulfur, and thereby converts the heme Fe^{++} in oxidized P-450 cam from low spin to high spin. On the other hand, 1-phenylimidazole may be an even more effective strong field ligand than cysteine and may replace the endogenous ligand by combining with the iron in oxidized P-450 cam. According to this view, reduced putidaredoxin is capable of reducing the high spin P-450 cam-camphor complex but not the low spin P-450 cam-phenylimidazole complex.

The model suggested by Peterson (20), however, does not explain how oxidized P-450 cam can be reduced when camphor is attached to the iron or how camphor is attached to the hemoprotein when oxygen is attached to the reduced iron. It therefore seems more likely that camphor causes the displacement of the strong field ligand indirectly, possibly by causing a conformational change around the heme. Such a conformational change, however, would have to impair the ability of P-450 cam to combine with type II substances, because the formation of the oxidized P-450 cam-ethylisocyanide complex is competitively inhibited by camphor.

The requirement of phospholipids by liver microsomal enzymes.—Coon and co-workers (26) have resolved solubilized preparations of the cytochrome P-450 system from liver microsomes into three components: cytochrome P-450, NADPH-cytochrome *c* reductase, and a lipid fraction. Recently it was found that the lipid fraction could be replaced by various phospholipids, of which phosphatidyl choline was the most effective (27). In contrast, phosphatidyl ethanolamine could not replace the lipid fraction and in fact inhibited the effect of phosphatidyl choline. The lipid fraction does not act by causing the polymerization of the cytochrome P-450 or by promoting the formation of a complex between cytochrome P-450 and NADPH-cytochrome *c* reductase since it does not cause an increase in the molecular weight of cytochrome P-450 (28). Nevertheless, the lipid fraction causes an increase in the reduction of cytochrome P-450 even though the lipid does not contain components that readily undergo redox reactions.

In attempting to corroborate the requirement for phospholipids by cyto-

chrome P-450 enzymes in liver microsomes, Chaplin & Mannering (29) were able to remove about 70% of the microsomal phospholipid phosphorous by treating liver microsomes with phospholipase *c*. Associated with the decrease in phospholipids was a 40% decrease in the metabolism of hexobarbital and ethylmorphine but only a 15% decrease in the metabolism of aniline. Moreover, after the treatment with phospholipase *c*, hexobarbital and ethylmorphine no longer caused a type I spectral change, but aniline caused an increase in the type II spectrum.

The meaning of these experiments of Chaplin & Mannering (29) is difficult to discern, since addition of phospholipids did not restore activity. Moreover, the impairment of the type I spectral change can be interpreted in several ways. One possibility is that the type I binding sites were destroyed. On the other hand, by analogy with the spectral changes observed with P-450 cam, the treatment by phospholipase *c* may have released a substance that combined with cytochrome P-450, thereby converting it to the type I form. In accord with the latter view, the treatment with phospholipase *c* increased the apparent K_m for ethylmorphine N-demethylation and increased the type II spectral change caused by aniline. Although the authors were able to show that phosphatidyl ethanolamine derivatives do not cause a type I spectral change, and that diglycerides do not inhibit the metabolism of aminopyrine or ethylmorphine, they could not unequivocally exclude the possible release of some other type I substance. Unfortunately, the releasing effect of butanol on endogenous substances (13) had not been discovered when these experiments were carried out.

Using another approach, Leibman & Estabrook (30) extracted liver microsomes with isooctane and found unusual spectral changes with hexobarbital. At low concentrations of hexobarbital, a diminished but typical type I spectral change was observed. As the concentration of hexobarbital was increased, the spectral change shifted to a type II. It is not clear, however, whether this anomalous behavior of the extracted microsomes with hexobarbital reflects the destruction of type I binding sites and the formation of a complex between hexobarbital and a hitherto unsuspected set of type II binding sites, or whether the treatment released endogenous type I substances which were displaced by hexobarbital thereby causing an "atypical" type II spectral change. In any event the authors were able to demonstrate that the effect was not due to isooctane per se since hexobarbital did not cause the anomalous spectral changes when isooctane was added to the experimental and the reference cuvettes.

Assays of Various Properties of Cytochrome P-450 Systems

As has been emphasized in various reviews (1–4, 31) on drug metabolism, the activity of the microsomal enzymes may be changed by a variety of treatments, including the removal of various endocrine glands, the administration of hormones, and the repeated administration of a diversity of drugs. In recent years, numerous studies have been carried out to determine

which components of the cytochrome P-450 system are altered by the different treatments. At first most of these studies were limited to the cytochrome P-450 content, but it soon became apparent that the activity of the cytochrome P-450 enzymes was not always directly proportional to the concentration of cytochrome P-450 in liver microsomes. For example, the marked species (32, 33) and sex differences (11, 34) in the metabolism of drugs, such as ethylmorphine, cannot be attributed to the relatively small differences in cytochrome P-450 content that are found among the various animal species and between the sexes of rat. Moreover, differences in the relative rates of metabolism of various drugs could not be solely due to differences in the cytochrome P-450 content of liver microsomes. For these reasons a battery of tests have been devised to elucidate which components of the system are altered by the various treatments.

Type I and type II spectral changes.—A number of studies have attempted to relate differences in the magnitude of type I and type II spectral changes caused by various drugs with differences in the rate of drug metabolism. But differences in the magnitude of these spectral changes are frequently difficult to interpret. For example, it is not clear whether the differences in the magnitude of the spectral change observed with various substrates are due to differences in the extinction coefficients of the cytochrome P-450 substrate complexes or to differences in their concentration. Moreover, many lipid-soluble endogenous substances, such as fatty acids (35, 36) and steroids (9, 37), may be highly bound to liver microsomal cytochrome P-450 and thus may be displaced by drugs, thereby interfering with the estimation of the magnitude of the spectral change. Indeed, by assuming that all cytochrome P-450 substrate complexes have the same extinction coefficient, Diehl et al (13) estimated that endogenous substrates may occupy as much as 20% of the total type I binding sites in freshly prepared liver microsomes. On storage of microsomes it is possible that the apparent loss of type I binding sites (38) may be due to fatty acids released from phospholipids and triglycerides by endogenous lipases. Furthermore, it is not clear whether aberrant spectral changes, such as the type II changes observed with hexobarbital and aminopyrine in certain microsomal preparations (30, 39), represent the displacement by the drug of an endogenous substrate from a cytochrome P-450 complex having an unusually high extinction coefficient, or whether the drugs can combine with both type I and type II sites, the relative proportions of which vary with the preparation. A few techniques have been developed to detect the presence of cytochrome P-450 complexes with endogenous type I substances. Ullrich and his colleagues have utilized the difference spectrum caused by iodine (40) or that caused by butanol (13). On the other hand, Jefcoate et al (41), have utilized the type II spectrum caused by octylamine to estimate the proportion of cytochrome P-450 present as the high spin form, which by analogy with P-450

cam should represent the cytochrome P-450 substrate (type I) complex. Unfortunately, these techniques have been used in only a few studies.

Ethylisocyanide difference spectrum.—After reduction of cytochrome P-450, ethylisocyanide causes the formation of peaks at about 455 nm and 430 nm in the difference spectrum (42), the relative magnitudes of which are dependent on the pH (43). For example, with microsomes from female rats the magnitudes of the peaks are equal at about pH 7.4, but the peak at 455 nm is higher than that at 430 nm at higher pH values and lower at lower pH values (44, 45). After treatment of rats with 3-methylcholanthrene, however, the magnitudes of the peaks are equal at about pH 6.8 (45). Since the absolute spectrum of cytochrome P-450 in liver microsomes from 3-methylcholanthrene-treated animals indicates the presence of a type I endogenous substrate (46), there has been considerable controversy in the literature as to whether the changes in the "cross-over pH" of the ethylisocyanide induced spectrum was due to the presence of the cytochrome P-450 substrate complex (39), or to the formation of a new type of cytochrome P-450 called cytochrome P_1-450 (45), cytochrome P-446 (46), or cytochrome P-448 (47) by various groups of workers. As discussed in another part of this review, there is now considerable evidence that 3-methylcholanthrene and other polycyclic hydrocarbons induce the formation of a new kind of cytochrome P-450. There is also evidence that the magnitude of the type I spectral change found with oxidized cytochrome P-450 does not parallel the magnitude of the peaks caused by ethylisocyanide with reduced cytochrome P-450 (48). Benzpyrene and hexobarbital, which cause type I spectral changes, do not significantly alter the ethylisocyanide-induced spectral changes (49). On the other hand, imipramine, which causes a relatively small type I spectral change, increases the ratio of the 455 nm/430 nm peaks caused by ethylisocyanide to a small but significant extent.[4] Moreover, with microsomes from 3-methylcholanthrene-treated rats, butanol markedly alters the absolute spectrum of cytochrome P-450 and changes the difference spectrum evoked by octylamine with oxidized cytochrome P-450 but does not alter the isocyanide induced spectral change with reduced cytochrome P-450.[3] Thus the ethylisocyanide induced spectral changes apparently provide information that is not readily attained by other techniques.

Extinction coefficients of cytochrome P-450-CO complexes.—The concept that liver microsomes contain different types of cytochrome P-450-CO raised the possibility that the CO complexes of these different types might have different extinction coefficients and that the mean extinction coefficients of the CO complexes might be altered by different treatments that alter the relative proportions of these forms.

At first it seemed likely that the various types of cytochrome P-450

[4] Sasame, H. A. and Gillette, J. R., unpublished observations.

might have markedly different extinction coefficients. In fact, using the offset method of Kinoshita & Hori (50), Hildebrandt et al (46) calculated that treatment of rabbits with phenobarbital caused the formation of a cytochrome P-450 whose CO complex had an absolute extinction coefficient of about 50 mM^{-1} cm^{-1}, whereas the treatment of rabbits with 3-methylcholanthrene caused the formation of a cytochrome P-450 whose CO complex had an absolute extinction coefficient of about 220 mM^{-1} cm^{-1}. It was soon recognized, however, that the offset method gave incorrect estimates of extinction coefficients for a number of reasons. In this method, the suspensions of liver microsomes from treated animals and the suspension from control animals are diluted so that the concentrations of cytochrome b_5 in all the suspensions are identical. After addition of dithionite and CO to the suspensions the difference in the absorbancies of the suspensions prepared from the treated animal and the control animal are measured and compared with the difference in heme content. Since differences between the suspensions in either the spectral change at 450 nm or the hemin content are frequently small, statistical variations in these measurements are usually high. The method also does not take into account differences in absorbancy caused by light scatter and by other substances in the microsomal suspensions. Moreover, it also assumes that the liver microsomes from the treated and control animals contain the same amount of the normal cytochrome P-450.

By measuring the difference in absorbancy at 450 nm between CO and N_2-treated microsomes according to the method of Omura & Sato (42) and correcting the total heme content for the heme of cytochrome b_5, Greene et al (51) found that neither phenobarbital nor 3-methylcholanthrene treatment of rabbits significantly affected the difference extinction coefficient of cytochrome P-450 in rabbit liver microsomes. Nevertheless, these authors showed that treatment of male rats with 3-methylcholanthrene increased the difference extinction coefficient from about 84 mM^{-1} cm^{-1} to about 102 mM^{-1} cm^{-1} but that treatment with phenobarbital did not affect the difference extinction coefficient.

By contrast Fujita & Mannering (52) have purified cytochrome P-450 in liver microsomes from phenobarbital- and 3-methylcholanthrene-treated rats and have found that the difference extinction coefficient of cytochrome P-450 from phenobarbital-treated rats was about 58 mM^{-1} cm^{-1}, whereas that from 3-methylcholanthrene was about 78 mM^{-1} cm^{-1}.

Stripp et al (53), observed a sex difference in the extinction coefficient of cytochrome P-450 in rat liver microsomes. Although the extinction coefficient of the hemoprotein in liver microsomes from 6 week old males was about 86 mM^{-1} cm^{-1}, its extinction coefficient in liver microsomes from 6 week old female rats was about 99 mM^{-1} cm^{-1}. Neither castration of male rats nor administration of 3-methyltestosterone to castrated male rats, however, altered the extinction coefficient.

The validity of these extinction coefficients remains in doubt. In all of these studies it has been assumed that the only hemoproteins in liver micro-

somes are cytochrome P-450 and cytochrome b_5. Recently, a cyanide-sensitive hemoprotein (54) has been isolated from liver microsomes, but neither the concentration nor the spectral properties of the possible CO complex of this hemoprotein is known.

Cytochrome P-450 reduction.—Since Gigon et al (11) found that the difference between the rates of cytochrome P-450 reduction in the presence and absence of ethylmorphine was about the same order of magnitude as the rate of ethylmorphine N-demethylation, they suggested that the rate-controlling step in the oxidation of type I substances is the rate of reduction of cytochrome P-450-substrate (type I) complex. In accord with this view, Holtzman (55) has reported that NADPD evoked similar isotope effects on cytochrome P-450 reduction and ethylmorphine metabolism. Moreover, Schenkman (56) found that the activation energies for aminopyrine demethylation and cytochrome P-450 reduction were similar. Type II substances, however, decelerated the reduction of cytochrome P-450, and thus the rate-controlling step of the metabolism of these substances is difficult to determine.

If the rate-controlling step in the metabolism of drugs is the reduction of the cytochrome P-450-complex, the finding that the rate of reduction of liver microsomal cytochrome P-450 is polyphasic can account for the finding that the K values for CO inhibition vary with the $CO:O_2$ ratio and differ with the substrate (57). The reason for this becomes clear when it is realized that the rapidly reduced form of cytochrome P-450 would not only account for most of the drug metabolism but also would be more sensitive to CO. In accord with this view Sasame found that ethylmorphine increases the steady-state level of the CO complex[5] (see also Narasimhulu, 58) and that the percent of inhibition of ethylmorphine N-demethylation by CO parallels the CO complex related to the rapidly reduced portion of the cytochrome P-450 rather than the total amount of cytochrome P-450 (see Gillette, 12). Studies on the rate of cytochrome P-450 reduction in the presence of substrates have thus proved useful in evaluating the effectiveness of the cytochrome P-450 system prior to the oxygenation step.

NADPH cytochrome c reductase.—At first glance, it might seem that changes in NADPH cytochrome *c* reductase activity would not affect the rate of oxidation of drugs, because the activity of this enzyme is an order of magnitude greater than either the rate of cytochrome P-450 reduction or the rate of drug oxidation (11). If NADPH-cytochrome *c* reductase reduces cytochrome P-450 directly and not through an intermediate electron carrier, however, the rate of reduction should depend on the concentrations of both the oxidized cytochrome P-450-substrate complex and the reduced NADPH-cytochrome *c* reductase, i.e., the reaction would be second order.

[5] Sasame, H. A. and Gillette, J. R., unpublished observations.

Thus the high NADPH-cytochrome *c* reductase activity merely indicates that under steady-state conditions most of the reductase is in its reduced form (about 80–90%) and not that the rate of cytochrome P-450 reduction is virtually independent of the total concentration of NADPH-cytochrome *c* reductase within the microsomes. Indeed as discussed below some of the alterations in the rate of cytochrome P-450 reduction have been shown to be due to changes in the microsomal content of this enzyme, as measured by its activity with cytochrome *c*.

Relationship between NADPH-oxidation and drug metabolism.—Since the mixed function oxidase mechanism assumes that equal amounts of NADPH, O_2, and drug are consumed in the reaction, it would seem logical that measuring the rate of NADPH-oxidation would be useful in determining the rate of drug metabolism without actually measuring the metabolites of the drug or the disappearance of the substrate. But the stoichiometric relationships predicted by the mixed function oxidase mechanism have rarely been demonstrated. The problem in obtaining the correct stoichiometry has been the difficulty in determining how much of the endogenous NADPH-oxidation is mediated by cytochrome P-450. If none of the endogenous NADPH-oxidation were mediated by the cytochrome P-450 system, subtraction of the endogenous rate of NADPH-oxidation from the rate in the presence of substrate would be sufficient. But the finding that CO inhibits the endogenous rates of NADPH-oxidation and the finding that the increase in NADPH-oxidation caused by the substrate is frequently less than the rate of drug metabolism suggest that a significant fraction of the endogenous NADPH-oxidation is mediated by cytochrome P-450. Recently Stripp et al (59) have devised techniques based on the effects of CO on drug metabolism and NADPH-oxidation to correct for the CO insensitive reactions. With these techniques they have obtained values with a number of substrates that closely approach the theoretical stoichiometry.

The stoichiometry of hexobarbital oxidation, however, is unusual in that the rate of hexobarbital oxidation by liver microsomes from rabbits (60) and female rats (12) is considerably less than the substrate dependent NADPH-oxidation. Under certain conditions, then, the metabolism of this drug becomes uncoupled from electron transport through cytochrome P-450.

An even more dramatic example of the uncoupling effect was shown by Ullrich & Diehl with perfluorohexane (61). This substance causes a type I spectral change with oxidized cytochrome P-450 in liver microsomes, and stimulates NADPH-oxidation, but is not metabolized at all.

Similarly, androstenedione stimulates NADPH-oxidation by adrenal mitochondria but does not undergo oxidation (62). Moreover, Narasimhulu (62) found that the ratio of the steroid dependent NADPH-oxidation to oxygen utilization was 2:1, suggesting that NADPH reduced the "active oxygen" cytochrome P-450 complex by an unknown mechanism.

It is obvious from these examples that claims of drug metabolism based solely on substrate dependent NADPH-oxidation should be interpreted with care.

Induction and Impairment of Cytochrome P-450 Enzyme Systems

Studies on various aspects of the cytochrome P-450 enzyme systems have revealed that changes in the rate of drug metabolism may be caused by alterations in any one of the components or properties of the system. Indeed, in many instances changes in drug metabolism may reflect alterations in several of the properties of the system. Moreover, the various treatments may affect the metabolism of drugs in subtle ways, and it is not always possible to correlate changes in the metabolism of one drug with changes in the metabolism of another. Indeed, some treatments seem to affect the metabolism of hexobarbital, aminopyrine, and ethylmorphine differently, even though these substrates are all type I substances.

Induction with phenobarbital.—Several studies have shown that treatment of animals with phenobarbital enhances the metabolism of foreign compounds by increasing the activity of NADPH-cytochrome *c* reductase and the concentration of cytochrome P-450. Phenobarbital also increases the magnitude of the type I and type II spectral changes per mg protein (9) and the rate of cytochrome P-450 reduction, whether related to microsomal protein or to the cytochrome P-450 content (63). Associated with the increase in cytochrome P-450, there is also an increase in δ-aminolevulinic acid synthetase and the synthesis of heme in liver (64, 65). As the level of cytochrome P-450 approaches steady-state after repeated administration of phenobarbital, the activity of the synthetase and the rate of heme synthesis increase rapidly during the first 16 hours, reach a plateau lasting for about 2 days and then decline (64). The fact that the rate of heme synthesis declines even though the cytochrome P-450 and total heme content in liver microsomes remain relatively constant after 2 days is consistent with the view of Marver (65) that a feed-back mechanism is brought into play.

The diet of animals can have a considerable influence on the induction by phenobarbital. When rats are fasted during treatment with the barbiturate, the levels of cytochrome P-450 are markedly increased (66), presumably because fasting decreases the breakdown of cytochrome P-450 (67). Moreover, McLean and his colleagues (68) have found that phenobarbital causes a greater increase in liver microsomal cytochrome P-450 in rats fed diets containing unsaturated fatty acids than in those fed diets containing protein and carbohydrate alone. But these effects may be due to the presence of antioxidants (69, 70) added to oils by the manufacturer or to peroxidized lipids and steroids (71), which are known to induce the drug metabolizing systems.

In the past, various studies led to the view that the inducing effects of

phenobarbital were not mediated by alterations in hormones (2–4, 31). This view was confirmed by the recent demonstration that phenobarbital induces microsomal enzymes in tissue culture cells derived from fetal liver (72).

Induction with polycyclic hydrocarbons.—Early studies on the mechanism of induction of microsomal enzymes showed that induction with polycyclic hydrocarbons differed from that with phenobarbital in a number of ways. Phenobarbital increased the metabolism of most of the drug substrates of the microsomal enzymes, whereas the polycyclic hydrocarbons increased the metabolism of relatively few of them, such as the N-demethylation of azo dyes and the hydroxylation of aniline and 3,4-benzpyrene (31). The simultaneous administration of phenobarbital and 3-methylcholanthrene produced additive effects on the metabolism of drugs whose metabolism was increased by either inducer, even when very large doses of inducers were used (73, 74); the additive effects of the inducers on 3,4-benzpyrene hydroxylation were recently confirmed with tissue culture cells (72). The polycyclic hydrocarbons increased the amount of cytochrome P-450 (45, 46) but did not increase the amount of NADPH-cytochrome *c* reductase (75, 76), whereas phenobarbital increased both of these components (75, 77, 78).

Treatment of animals with 3-methylcholanthrene causes changes in a number of other properties of the cytochrome P-450 systems in liver microsomes. Although the effects of SKF 525-A on ethylmorphine N-demethylation are not changed, presumably because 3-methylcholanthrene does not induce the enzyme that catalyzes this reaction, the K_I of SKF 525-A for azo-dye demethylation is markedly increased (79). On the other hand, 7,8-benzoflavone, which has little effect on 3,4-benzpyrene hydroxylase in liver microsomes from untreated animals, markedly inhibits the enzyme in liver microsomes from 3-methylcholanthrene-treated animals (80).

At first it seemed possible that the differential effects of 3-methylcholanthrene might be due to the binding of the inducer or one of its metabolites to cytochrome P-450, because the absolute spectrum of the hemoprotein in liver microsomes from treated animals showed a peak at 390 nm (46), which is analogous to the type I spectrum observed with substrates, such as ethylmorphine, hexobarbital, and aminopyrine. But a number of facts make this explanation unlikely: (*a*) The changes in the spectrum of the cytochrome P-450-ethylisocyanide complex observed by Sladek & Mannering (45) cannot be demonstrated by addition of 3-methylcholanthrene or hexobarbital to liver microsomes (49). (*b*) The change in the ethylisocyanide spectrum does not occur immediately after injection of 3-methylcholanthrene but develops with time and becomes maximal at about a day after injection of the inducer (81). Moreover, the alteration of the spectrum of the ethylisocyanide complex can be prevented by inhibitors of protein synthesis, such as ethionine and actinomycin D (82). (*c*) Fujita & Mannering (52) have isolated the cytochrome P-450 from liver microsomes of animals receiving 3-methylcholanthrene and have shown that it does not combine

with hexobarbital, whose metabolism is not induced, even though the preparation did not have a peak at 390 nm. (*d*) Lu et al (83), have isolated the cytochromes from liver microsomes of rats treated with either phenobarbital or 3-methylcholanthrene and have shown that the preparations exhibit different substrate specificities in reconstituted enzyme systems; as expected, the hemoprotein from phenobarbital-treated rats preferentially catalyzes the 16 α-hydroxylation of testosterone and the N-demethylation of benzphetamine, whereas the hemoprotein from 3-methylcholanthrene-treated rats preferentially catalyzes the hydroxylation of 3,4-benzpyrene. By contrast both hemoproteins are effective in catalyzing the 7 α-hydroxylation of testosterone and the N-demethylation of chlorcyclizine.

It is noteworthy that the inducing effects of 3-methylcholanthrene differ markedly among various strains of mice (84). In C57BL/CN mice, it markedly induces the hydroxylation of 3,4-benzpyrene hydroxylase, but in DBA/2N mice it has little or no effect on this enzyme. Studies with various F_1 and F_2 hybrids of these strains indicate that the ability of 3-methylcholanthrene to induce this microsomal enzyme follows autosomal dominant inheritance.

Sex differences in drug metabolism by rat liver microsomes.—Liver microsomes from male rats metabolize a wide variety of drugs more rapidly than do those from female rats (85, 86). However, liver microsomes from male and female rats contain about the same amount of NADPH-cytochrome *c* reductase (87) and cytochrome P-450 (11, 87). Moreover, there are only minor differences in the endogenous cytochrome P-450 reductase (11, 88). However, the magnitude of the type I spectral changes caused by aminopyrine, hexobarbital (34), and ethylmorphine (87) is considerably greater with liver microsomes from male rats than with those from female rats. Moreover, these substances stimulate cytochrome P-450 reduction to a greater extent in microsomes from males than in those from females (11, 88).

The sex difference in the magnitude of type I spectral change per cytochrome P-450 suggests the presence of different kinds of cytochrome P-450 in liver microsomes. In accord with this view, the "cross-over pH" of the ethylisocyanide-cytochrome P-450 complex is 7.4 with liver microsomes from females and 8.2 with liver microsomes from males (44). Moreover, the extinction coefficient of cytochrome P-450 is about 10% lower in liver microsomes from males than in those from females (53).

Hamrick et al (89) have found a sex difference in the relationship between NADPH-oxidation and ethylmorphine N-demethylation. Using the techniques developed by Stripp et al (59) they found that the ratio of the rate of ethylmorphine N-demethylation to the rate of NADPH-oxidized through the cytochrome P-450 system in the presence of substrate approached 1:1 in liver microsomes from male rats but was only 0.5:1 in those from female rats.

Treatment of female rats with methyltestosterone causes an increase not only in ethylmorphine N-demethylase activity, but also in the magnitude of the type I spectral change and in the ratio of the rates of ethylmorphine N-demethylation and NADPH-oxidation. But the treatment with methyltestosterone did not affect the level of cytochrome P-450 or the activity of endogenous NADPH-oxidation.

Effects of adrenalectomy and glucocorticoids.—Adrenalectomy of rats decreases the activity of the liver microsomal enzymes that catalyze the oxidation of type I substrates, such as hexobarbital (90, 91), aminopyrine (91), and ethylmorphine (92), the decrease in activity being greater in males than in females (91). But adrenalectomy of male rats does not significantly change the levels of cytochrome P-450 (92). Although the impairment of hexobarbital metabolism is apparently related to a decrease in the magnitude of the hexobarbital-induced type I spectral change per cytochrome P-450 (93), the impairment of ethylmorphine demethylation is not accompanied by a decrease in the ethylmorphine-induced spectral change (92). However, adrenalectomy of male rats decreases the activities of NADPH-cytochrome *c* reductase, cytochrome P-450 reductase, and ethylmorphine N-demethylase to about the same extent (92). Moreover, the administration of cortisone to adrenalectomized male rats reverses the effects of adrenalectomy not only on the reductase activities but also on the ethylmorphine N-demethylase activity (92). These findings suggest that the mechanism of impairment of the drug metabolizing enzyme system caused by adrenalectomy depends on the substrate. It is also noteworthy that adrenalectomy of mice and rabbits has little effect on hexobarbital metabolism by liver microsomes, possibly because these species do not manifest sex differences in hexobarbital metabolism (93).

Spironolactone administration.—Recently, Solymoss et al (94) demonstrated that spironolactone, a steroid that has little or no hormonal activity, stimulates the metabolism of drugs in female rats. In accord with these findings, Stripp et al (95) have shown that spironolactone treatment of female rats increases the metabolism of ethylmorphine, hexobarbital, and benzpyrene by liver microsomes. Associated with these increases in enzyme activity was an increase in NADPH-cytochrome *c* reductase but little if any change in the type I spectrum caused by ethylmorphine. Moreover, the cytochrome P-450 content was not increased; indeed it tended to decrease. The treatment with spironolactone did not affect cytochrome P-450 reduction in the absence of drug substrate despite the increase in NADPH-cytochrome *c* reductase. But it did increase the substrate-dependent cytochrome P-450 reduction. By contrast, the treatment increased both the endogenous and the substrate-stimulated NADPH-oxidation by liver microsomes and increased the ratio of ethylmorphine N-demethylation to NADPH-oxidized.

Stripp et al (95) also found a sex difference in the effects of spironolac-

tone in rats that could not be explained by changes in any of the components of the enzyme system. Although in male rats, spironolactone caused a relatively small increase in ethylmorphine N-demethylation, it decreased the oxidation of hexobarbital and 3,4-benzpyrene. Like its effects in females, however, spironolactone in males did not affect type I spectral changes caused by ethylmorphine and hexobarbital, but caused a small decrease in cytochrome P-450 content and an increase in NADPH-cytochrome *c* reductase. Moreover, spironolactone in males increased the substrate dependent cytochrome P-450 reduction, but not its endogenous reduction, and it increased both the endogenous and substrate-dependent NADPH-oxidation. These findings thus indicate that spironolactone treatment paradoxically enhances the hexobarbital-induced electron flux through the cytochrome P-450 system and at the same time decreases hexobarbital metabolism.

In contrast to its effects in rats, spironolactone in mice increases the amount of cytochrome P-450 in liver microsomes, but the increase does not entirely account for the increase in the metabolism of hexobarbital or ethylmorphine (96–98).

Impairment of drug metabolism by CCl_4.—The oral administration of CCl_4 impairs the metabolism of drugs both in vivo (99) and in vitro (99–103). Within the first 3 hours after CCl_4 administration there is a decrease in cytochrome P-450, as measured by a decrease in either the absorbancy at 450 nm (101–103) or the heme content (104). At this time, there is no decrease in either NADPH-cytochrome *c* reductase or cytochrome b_5, suggesting that the impairing effects on cytochrome P-450 are rather selective.

Although these impairing effects of CCl_4 may be delayed by the simultaneous administration of SKF 525-A (102), an inhibitor of drug oxidation by liver microsomes, they are not affected by other potent inhibitors, such as Lilly 18947 (2,4-dichloro-6-phenylphenoxyethyl diethylamine), DPEA (2,4-dichloro-6-phenylphenoxyethylamine) or SKF 26754-A (aminoethyl diphenylpropylacetate) (105). Thus the delay in the impairing effects of CCl_4 by SKF 525-A may not be completely due to a blockade of conversion of CCl_4 to an active metabolite but to another mechanism. Indeed Marchand et al (106) have suggested that SKF 525-A may exert its protective effects by slowing the absorption of CCl_4 from the intestine.

Formation of a Metabolite by Different Enzymes

One of the complications in studying the metabolism of drugs is that a metabolite may be formed by more than one enzyme or along different pathways.

Pathways of N-dealkylation.—During the past several years, two different mechanisms for the N-dealkylation of foreign compounds have gained acceptance. In the first mechanism, cytochrome P-450 catalyzes the C-hydroxylation of a tertiary (or secondary) amine to form an unstable inter-

mediate that decomposes with the formation of a secondary (or primary) amine and an aldehyde (1). In the second mechanism, a flavoprotein catalyzes the conversion of a tertiary amine to its N-oxide or of a secondary amine to its hydroxylamine (107); cytochrome P-450 then catalyzes the rearrangement and decomposition of the respective intermediates to form an aldehyde and the corresponding amines (108). Since cytochrome P-450 is a component of both mechanisms, the fact that carbon monoxide and SKF 525-A block the formation of the final product is not surprising and thus the relative importance of the two mechanisms has been difficult to assess when only the final products are measured. However, the formation of the N-oxide via the NADPH-dependent flavoprotein is not inhibited by CO or by SKF 525-A and thus the N-oxide accumulates in the incubation mixtures (109). Although it may seem possible that the substrate-stimulated NADPH oxidation in the presence and absence of CO might be used to assess the relative importance of the N-oxidation and the C-oxidation pathways, the finding of Stripp et al (59) that high substrate concentrations change the stoichiometry between drug metabolism and the electron transport through cytochrome P-450 complicates the interpretation of such data. Moreover, the finding that N-oxides may be reduced to amines in liver preparations causes further difficulties in assessing the importance of the N-oxide mechanism (110). Clearly, other approaches, such as the use of specific antibodies to NADPH cytochrome *c* reductase (Masters et al, 111) are needed to differentiate between the two mechanisms. It should be kept in mind, however, that there probably are a number of enzymes that catalyze N-hydroxylation reactions, because the N-oxidase isolated by Ziegler's group does not catalyze the hydroxylation of primary aromatic amines or their acetylated analogs (112).

Mechanisms of aromatic hydroxylation.—It has been suspected for many years that the conversion of certain aromatic compounds, such as naphthalene, to GSH conjugates and dihydro-diols, is mediated by epoxide intermediates (113). In accord with this view only one of the oxygen atoms incorporated into the dihydro-diol of naphthalene comes from atmospheric oxygen (114) and radiolabeled naphthalene epoxide can be detected after incubation of liver microsomes with NADPH, radiolabeled naphthalene, and unlabeled naphthalene epoxide (115). Moreover the conversion of epoxides to dihydro-diol derivatives is catalyzed by microsomal enzymes called epoxide hydrases (116–119).

Since aromatic epoxides in water decompose spontaneously to phenols (115), hydroxylation of aromatic compounds by liver microsomal systems conceivably can occur either directly or indirectly by way of epoxide intermediates. To evaluate this possibility Jerina et al (115) showed that GSH inhibits the formation by liver microsomes of naphthol and naphthalene dihydrol-diol from naphthalene, presumably by channeling the epoxide toward the formation of a GSH conjugate of naphthalene. Similarly, Zampaglione

et al (120) found that GSH also inhibited the conversion of bromobenzene to its dihydro-diol and phenolic metabolites.

Although these findings raise the possibility that the hydroxylation of all aromatic compounds is mediated by epoxides, it should be pointed out that Jerina et al (115) failed to detect radiolabeled benzene epoxide after incubation of liver microsomes with radiolabeled benzene and unlabeled benzene epoxide even though considerable amounts of radiolabeled phenol were formed.

Whether all hydroxylation reactions are mediated by epoxide formation is of considerable importance, because it has been suggested that epoxides of various aromatic polycyclic hydrocarbons and halogenated benzenes mediate a diversity of toxicities including mutagenesis (121), carcinogenesis (122), and liver necrosis (123).

Deamination of amphetamine.—Shortly after the discovery that rabbit liver microsomes catalyze the deamination of amphetamine, Brodie et al (1) proposed that the carbon adjacent to the nitrogen is hydroxylated to form an unstable carbinolamine, which spontaneously rearranges to form phenylacetone and ammonia. However, Hucker et al (124) recently found that the major metabolite of amphetamine formed by rabbit liver microsomes is phenylacetone oxime and speculated that amphetamine is converted first to phenylacetone imine which in turn is hydroxylated to the oxime and that the oxime is hydrolyzed to phenylacetone. Since the mechanism proposed by Brodie et al (1) predicts that all of the oxygen in phenylacetone originates from the atmosphere, whereas that of Hucker et al (124) predicts that none originates from the atmosphere, the finding of Parli et al (125) that about 25–30% of the oxygen in phenylacetone comes from the atmosphere suggests that both mechanisms occur with rabbit liver microsomes. The mechanism by which phenylacetone oxime is formed remains obscure. Smith & Dring (126) suggested that amphetamine is dehydrogenated to the imine, which in turn is either hydroxylated to phenylacetone or hydroxylated to the oxime. It seems more likely to Parli et al (125) and to us, however, that the imine is formed by way of the carbinolamine.

In any event it now seems likely that the oxidative deamination of isoalkylamines may be formed by either the rearrangement of carbinolamines or the hydrolysis of the oxime-intermediates, and that the relative importance of the two pathways will depend on the substrate and the animal species.

Reduction of nitro- and azo-compounds.—Liver contains a host of enzymes that catalyze the reduction of nitro- and azo-compounds. The relative importance of these enzymes, however, depends on the substrate and the animal species.

p-Nitrobenzoic acid is reduced to *p*-aminobenzoic acid by microsomal cytochrome P-450 (127) or to hydroxylaminobenzoic acid by an unidentified enzyme in liver supernatant (128), but is not reduced to any significant ex-

tent by NADPH cytochrome *c* reductase (127) in liver microsomes or by xanthine oxidase (129, 130) in liver supernatant. By contrast niridazole and a number of nitrofurans are reduced by NADPH-cytochrome *c* reductase in liver microsomes (131) and by xanthine oxidase in liver supernatant (130) but not to any significant extent by cytochrome P-450 (131). Moreover, nitroquinoline N-oxide is reduced by a dicoumarol sensitive enzyme in liver supernatant, but not to any significant extent by other liver enzymes (132).

The reduction of azo-compounds is even more confusing. For example, Neoprontosil is reduced by both NADPH-cytochrome *c* reductase and cytochrome P-450 in liver microsomes (75, 133), but owing to the difference in the Km values the relative importance of these enzymes depends on the substrate concentration (134). At low concentrations of Neoprontosil, the cytochrome P-450 catalyzed reaction accounts for virtually all of the reduction of the substrate, but at high substrate concentrations, the cytochrome *c* reductase reaction predominates. By contrast phenylazopyridine is reduced almost entirely by NADPH-cytochrome *c* reductase (135).

Although reduced cytochrome P-450 is capable of reducing a number of nitro- and azo-compounds because of its unusually low redox potential (136), it probably does not function as a reductase in vivo to any significant extent. As discussed above, the rate limiting step in the oxidation of drugs by cytochrome P-450 is probably the reduction of the cytochrome P-450 substrate complex. Thus most of the cytochrome P-450 exists in the oxidized form under steady-state conditions in air. Moreover, the intermediates in the reduction of nitro- and azo-reduction are frequently autoxidizable and thus the primary amine metabolites are not rapidly formed in vivo. For these reasons, most of the nitro- and azo-reduction observed in vivo is probably due to bacterial flora in the anaerobic environment of the gut.

Alcohol oxidation.—It is well known that alcohols are oxidized to ketones and aldehydes by a number of NADPH and NADH-dependent dehydrogenases (137). In recent years, however, considerable attention has been focused on the oxidation of ethanol by liver microsomal preparations, i.e. by a microsomal ethanol oxidizing system (MEOS). Depending on the purity and the handling of these preparations, however, the oxidation of ethanol can be due to a number of enzyme systems. Unwashed preparations of liver microsomes can contain alcohol dehydrogenase (138, 139). But these preparations also contain other ethanol oxidizing systems, because pyrazole, which inhibits alcohol dehydrogenase, does not totally inhibit ethanol oxidation (139). By analogy with the observation of Gillette et al (140) that NADPH oxidase in liver microsomes formed a peroxide (either hydrogen peroxide or a lipid hydroperoxide) that oxidized methanol in the presence of catalase, a part of the ethanol oxidation probably is due to the concerted action of NADPH oxidase and catalase. In accord with this view, Carter & Isselbacher (139) found that ethanol oxidation is partially blocked either by cyanide (which inhibits catalase) or by CO (which inhibits cytochrome P-

450 mediated NADPH oxidation). Nevertheless, Lieber and co-workers (141, 142) have reported that in their microsomal preparations, ethanol oxidation is not blocked by pyrazole, nor by the catalase inhibitors, cyanide and 4-amino-1,2,4-triazole. These workers thus suggest that ethanol can be oxidized directly by a cytochrome P-450 system rather than indirectly through a peroxide-catalase. However, these workers also reported that the activity of the system is not induced by either phenobarbital or 3-methylcholanthrene even though it is increased by treatment of the animals with ethanol. The mechanism of the so-called MEOS thus remains to be clarified.

Regardless of the identity of the MEOS there is little doubt that it plays a relatively minor role in the metabolism of ethanol in vivo. At best, it can account for only about 20–25% of the total ethanol metabolism in vivo (142) and thus even a marked alteration in the activity of MEOS would not alter the rate of ethanol metabolism to a clinically significant extent.

In Vivo Aspects of Drug Metabolism

It is frequently difficult to extrapolate data obtained from in vitro experiments to the living animal. Even when the investigator is interested only in relating the activity of the drug metabolizing enzymes in liver to the biological half-life of the drug in the animal, a number of problems may arise which are difficult or even impossible to evaluate with liver preparations alone. Gillette (143) has recently discussed some of these problems, including the effect of competitive inhibitors, the determination of rate limiting steps, the importance of the hepatic blood flow in limiting drug metabolism, and the factors that determine the volume of distribution of drugs. With in vivo studies it is sometimes difficult to determine whether the increase in drug metabolism after phenobarbital treatment of animals is due mainly to the induction of the cytochrome P-450 enzymes, an increase in hepatic blood flow (144, 145), or an increase in bile flow (146). It is also sometimes difficult to determine whether SKF 525-A slows drug metabolism by inhibiting cytochrome P-450 and other drug metabolizing enzymes, by slowing hepatic blood flow (145), by slowing the absorption of drugs from the gastrointestinal tract (106), or by a combination of all three.

The problems of relating in vitro enzyme activity to the pharmacologic or toxicologic effects of foreign compounds become even more difficult when pharmacologically inactive substances are converted to pharmacologically active metabolites.

In these instances it is impossible to decide a priori whether an inducer or inhibitor would lead to enhanced or diminished activity, because inducers and inhibitors may affect the rate of catabolism of the active metabolite as well as its rate of formation. For example, an inducer may increase the concentration of an active metabolite when the enzymes that catalyze its formation are induced to a greater extent than the enzymes that catalyze its destruction. On the other hand, an inducer may diminish the concentration of the active metabolite when the enzymes that catalyze its formation are

induced to a lesser extent than the enzymes that catalyze its destruction. Thus it should not be surprising that two different kinds of inducers cause completely opposite effects.

It is also important to realize that the concentration of endogenous cosubstrates may change as a drug is being conjugated, and that the pattern of drug metabolism can be altered when the cosubstrate is depleted from the cell. For example, high doses of salicylamide tend to deplete the body stores of sulfate; thus the rate-limiting step of salicylamide sulfate formation becomes the formation of sulfate from cysteine (147). Similarly, it is thought that acetaminophen prevents the periportal hepatotoxicity caused by N-acetyl-N-hydroxyaminofluorene by depleting the body of sulfate, thereby preventing the formation of the N-hydroxyamino derivative, an extraordinarily potent alkylating agent (148).

It should also be pointed out that the liver is composed of a heterogenous group of parenchymal cells having widely different enzyme activities. For example, histochemical studies have shown that 3,4-benzpyrene hydroxylase activity of liver is concentrated in the centrolobular regions of liver (149). Moreover, the administration of phenobarbital causes proliferation of the endoplasmic reticulum predominately in the centrolobular regions of liver and thus the heterogeneity in the distribution of the enzyme becomes even more marked after induction (150).

The importance of these points was dramatically illustrated to us by a series of studies on the mechanism of centrolobular necrosis caused by bromobenzene in liver. It now seems likely that bromobenzene causes centrolobular necrosis by being converted to its epoxide, which in turn becomes covalently bound to macromolecules in liver (123). In accord with this view, pretreatment of mice (or rats) with phenobarbital increases both the metabolism of bromobenzene in vivo and the centrolobular necrosis, whereas the administration of SKF 525-A (β-diethylaminoethyl diphenylpropylacetate) decreases both the metabolism of bromobenzene and the necrosis. Moreover, autoradiographs of paraffin sections of liver after ^{14}C-bromobenzene administration to mice showed that the covalently bound radiolabel was localized in the necrotic areas. Furthermore, covalent bonding of radiolabeled bromobenzene was demonstrated chemically. The amount of covalently bound bromobenzene, however, was low during the first few hours and then increased exponentially until maximal amounts were obtained at 12 to 24 hours (151). Most of the covalently bound bromobenzene occurred only after glutathione was decreased in liver (152), but the concentration of glutathione required to block covalent bonding in vitro is even lower than the lowest levels of glutathione per gram of liver found after bromobenzene administration (153). It therefore seems likely that GSH becomes completely depleted only in the centrolobular regions, where necrosis takes place. In other regions, the formation of the epoxide is too slow to deplete the cells of GSH and thus these regions do not become necrotic.

At first we were surprised to find that the administration of 3-methyl-

cholanthrene to rats completely prevented the hepatoxicity of bromobenzene (154), because pretreatment with 3-methylcholanthrene slightly increased bromobenzene metabolism (155). But analysis of the bromobenzene metabolites in urine showed that the treatment also increased the formation of bromobenzene dihydrodiol (155). These results thus suggested that after the parenchymal cells in the centrolobular region are depleted of GSH, bromobenzene epoxide can now be inactivated by epoxide hydrase, thereby preventing covalent bonding and the centrolobular necrosis. This interplay between the activity of the GSH conjugation system, the epoxide hydrase, and the activity of the cytochrome P-450 system that forms bromobenzene epoxide thus determines the severity and the localization of the liver necrosis.

One of the authors (D.C.D.) is a Research Associate in the Pharmacology-Toxicology program, National Institutes of General Medical Sciences, National Institutes of Health, Bethesda, Maryland.

LITERATURE CITED

1. Brodie, B. B., Gillette, J. R., La Du, B. N. 1958. *Ann. Rev. Biochem.* 27:427–54
2. Gillette, J. R. 1963. *Progr. Drug Res.* 6:11–73
3. Gillette, J. R. 1966. *Advan. Pharmacol.* 4:219–61
4. Gillette, J. R., et al, eds. 1969. *Microsomes and Drug Oxidation,* New York: Academic. 547 pp.
5. Mason, H. S. 1957. *Advan. Enzymol.* 19:79–233
6. Hayaishi, O. 1962. *Oxygenases,* ed. O. Hayaishi, 1–29. Academic: New York. 588 pp.
7. Remmer, H. et al. 1966. *Mol. Pharmacol.* 2:187–90
8. Imai, Y., Sato, R. 1966. *Biochem. Biophys. Res. Commun.* 22:620–26
9. Schenkman, J. B., Remmer, H., Estabrook, R. W. 1967. *Mol. Pharmacol.* 3:113–23
10. Jefcoate, C. R. E., Gaylor, J. L., Calabrese, R. L. 1969. *Biochemistry* 8:3455–63
11. Gigon, P. L., Gram, T. E., Gillette, J. R. 1969. *Mol. Pharmacol.* 5: 109–22
12. Gillette, J. R. 1971. *Metabolism* 20: 215–27
13. Diehl, H., Schädelin, J., Ullrich, V. 1970. *Z. Physiol. Chem.* 351:1359–71
14. Correia, M. A., Mannering, G. J. 1971. *Pharmacologist* 13:223
15. Oshino, N., Sato, R. 1971. *J. Biochem.* 69:169–80
16. Hildebrandt, A., Estabrook, R. W. 1971. *Arch. Biochem. Biophys.* 143:66–79
17. Estabrook, R. W., Hildebrandt, A. G., Baron, J., Netter, K. J., Leibman, K. 1971. *Biochem. Biophys. Res. Commun.* 42:132–39
18. Sasame, H. A. 1971. *Fed. Proc.* 30: 505
19. Katagiri, M., Gongali, B. N., Gunsalus, I. C. 1968. *J. Biol. Chem.* 243:3543–46
20. Peterson, J. A. 1971. *Arch. Biochem. Biophys.* 144:678–93
21. Omura, T., Sanders, E., Estabrook, R. W., Cooper, D. Y., Rosenthal, O. 1966. *Arch. Biochem. Biophys.* 117:660–73
22. Gunsalus, I. C. 1968. *Z. Physiol. Chem.* 349:1610–13
23. Griffin, B., Peterson, J. A. 1971. *Arch. Biochem. Biophys.* In press
24. Peterson, J. A., Ishimura, Y., Baron, J., Estabrook, R. W. 1971. *Int. Symp. Oxidases Oxygenases,* eds. H. S. Mason, T. King, M. Morrison. Wiley: New York. In press
25. Ishimura, Y., Ullrich, V., Peterson, J. A. 1971. *Biochem. Biophys. Res. Commun.* 42:140–46
26. Lu, A. Y. H., Coon, M. J. 1968. *J. Biol. Chem.* 243:1331–32
27. Strobel, H. W., Lu, A. Y. H., Heidema, J., Coon, M. J. 1970. *J. Biol. Chem.* 245:4851–54
28. Autor, A. P., Strobel, H. W., Heidema, J., Coon, M. J. 1971. *Fed. Proc.* 30:505
29. Chaplin, M. D., Mannering, G. J.

1970. *Mol. Pharmacol.* 6:631–40
30. Leibman, K. C., Estabrook, R. W. 1971. *Mol. Pharmacol.* 7:26–32
31. Conney, A. H. 1967. *Pharmacol. Rev.* 19:317–66
32. Davies, D. S., Gigon, P. L., Gillette, J. R. 1968. *Biochem. Pharmacol.* 17:1865–72
33. Kato, R., Onoda, K., Takayanagi, M. 1970. *Jap. J. Pharmacol.* 20:157–63
34. Schenkman, J. B., Frey, I., Remmer, H., Estabrook, R. W. 1967. *Mol. Pharmacol.* 3:516–25
35. DiAugustine, R. P., Fouts, J. R. 1969. *Biochem. J.* 115:547–54
36. Das, M. L., Orrenius, S., Ernster, L. 1968. *Eur. J. Biochem.* 4:519–23
37. Kuntzman, R., Jacobson, M., Schneidman, K., Conney, A. H. 1964. *J. Pharmacol. Exp. Ther.* 146:280–85
38. Hewick, D. S., Fouts, J. R. 1970. *Biochem. Pharmacol.* 19:457–72
39. Schenkman, J. B., Greim, H., Zange, M., Remmer, H. 1969. *Biochim. Biophys. Acta* 171:23–31
40. Ullrich, V. 1969. *Z. Physiol. Chem.* 350:357–65
41. Jefcoate, C. R. E., Calabrese, R. L., Gaylor, J. L. 1970. *Mol. Pharmacol.* 6:391–401
42. Omura, T., Sato, R. 1964. *J. Biol. Chem.* 239:2379–85
43. Imai, Y., Sato, R. 1966. *Biochem. Biophys. Res. Commun.* 23:5–11
44. El Defrawy, S. A. M. 1967. *Studies on the sex-dependent difference in drug metabolism.* Ph.D. thesis. Univ. Minnesota, Minneapolis, 117 pp.
45. Sladek, N. E., Mannering, G. J. 1966. *Biochem. Biophys. Res. Commun.* 24:668–74
46. Hildebrandt, A. G., Remmer, H., Estabrook, R. W. 1968. *Biochem. Biophys. Res. Commun.* 30:607–12
47. Alvares, A. P., Schilling, G., Levin, W., Kuntzman, R. 1968. *Biochem. Biophys. Res. Commun.* 29:521–26
48. Mannering, G. J. 1971. *Metabolism* 20:228–45
49. Alvares, A. P., Schilling, G., Levin, W., Kuntzman, R. 1971. *J. Pharmacol. Exp. Ther.* 176:1–10
50. Kinoshita, T., Horie, S. 1967. *J. Biochem.* 61:26–34
51. Greene, F. E., Stripp, B., Gillette, J. R. 1971. *Pharmacology* 5:43–48
52. Fujita, T., Mannering, G. J. 1971. *Chem. Biol. Interactions* 3:264–65
53. Stripp, B., Greene, F. E., Gillette, J. R. 1971. *Pharmacology.* In press
54. Gaylor, J. L., Moir, N. J., Seifried, H. E., Jefcoate, C. R. E. 1970. *J. Biol. Chem.* 245:5511–13
55. Holtzman, J. L. 1970. *Biochemistry* 9:955–1000
56. Schenkman, J. B. 1971. *Chem. Biol. Interactions* 3:306–07
57. Estabrook, R. W., Franklin, M. R., Hildebrandt, A. G. 1970. *Ann. NY Acad. Sci.* 174:218–32
58. Narasimhulu, S., Cooper, D. Y., Rosenthal, O. 1966. *Fed. Proc.* 25: 282
59. Stripp, B., Zampaglione, N. G., Hamrick, M. E., Gillette, J. R. 1971. *Mol. Pharmacol.* In press
60. Gillette, J. R. 1964. *Drugs and Enzymes: Proc. 2nd Int. Pharmacol. Meet.,* eds. B. B. Brodie, J. R. Gillette, 9–22. Macmillan:New York 504 pp.
61. Ullrich, V., Diehl, H. 1971. *Eur. J. Biochem.* 20:509–12
62. Narasimhulu, S. 1971. *Fed. Proc.* 30 (3, Part II):1091
63. Gnosspelius, Y., Thor, H., Orrenius, S. 1969. *Chem. Biol. Interactions* 1:125–37
64. Tephly, T. R., Hasegawa, E., Baron, J. 1971. *Metabolism* 20:200–14
65. Marver, H. S. See Ref. 4, 495–515
66. Kato, R., Gillette, J. R. 1963. *Pharmacologist* 5:240
67. Greim, H. 1970. *Arch. Pharmakol. Exp. Pathol.* 266:261–75
68. Marshall, W. J., McLean, A. E. M. 1971. *Biochem. J.* 122:569–73
69. Gilbert, D., Golberg, L. 1965. *Food Cosmet. Toxicol.* 3:417–32
70. Gilbert, D. 1969. *Biochem. J.* 115: 59P
71. Brown, R. R., Miller, J. A., Miller, E. C. 1954. *J. Biol. Chem.* 209: 211–22
72. Gielen, J. E., Nebert, D. W. 1971. *Science* 172:167–69
73. Gram, T. E., Gillette, J. R. 1971. *Fundamentals of Biochemical Pharmacology,* ed. Z. M. Bacq, 571–609. Pergamon Press:Oxford
74. Bidleman, K., Mannering, G. J. 1970. *Mol. Pharmacol.* 6:697–701
75. Hernandez, P. H., Mazel, P., Gillette, J. R. 1967. *Biochem. Pharmacol.* 16:1877–88

76. von der Decken, A., Hultin, T. 1960. *Arch. Biochem. Biophys.* 90:201–07
77. Orrenius, S., Ericsson, J. L. E., Ernster, L. 1965. *J. Cell Biol.* 25: 627–39
78. Remmer, H., Merker, H. J. 1965. *Ann. NY Acad. Sci.* 123:79–97
79. Mannering, G. J., Sladek, N. E., Parli, C. J., Shoeman, D. W. See Ref. 4, 303–30
80. Wiebel, F. J., Leutz, J. C., Diamond, L., Gelboin, H. V. 1971. *Arch. Biochem. Biophys.* 144:78–86
81. Alvares, A. P., Parli, C. J., Mannering, G. J. 1967. *Pharmacologist* 9:203
82. Alvares, A. P., Schilling, G., Levin, W., Kuntzman, R. 1968. *J. Pharmacol. Exp. Ther.* 163:417–24
83. Lu, A. Y. H., Kuntzman, R., West, S., Jacobson, M., Conney, A. H. 1971. *Pharmacologist* 13:222
84. Nebert, D. W., Goujon, F. M., Gielen, J. E. 1971. *Pharmacologist* 13:222
85. Quinn, G. P., Axelrod, J., Brodie, B. B. 1958. *Biochem. Pharmacol.* 1: 152–59
86. Kato, R., Gillette, J. R. 1965. *J. Pharmacol. Exp. Ther.* 150:279–84
87. Davies, D. S., Gigon, P. L., Gillette, J. R., 1969. *Life Sci.* 8 (Part II): 85–91
88. Gigon, P. L., Gram, T. E., Gillette, J. R. 1969. *Biochem. Biophys. Res. Commun.* 31:558–62
89. Hamrick, M. E., Zampaglione, N. G., Stripp, B., Gillette, J. R. In preparation
90. Remmer, H. 1958. *Arch. Exp. Pathol. Pharmakol.* 233:184–91
91. Kato, R., Gillette, J. R. 1965. *J. Pharmacol. Exp. Ther.* 150:285–91
92. Castro, J. A., Greene, F. E., Gigon, P. L., Sasame, H., Gillette, J. R. 1970. *Biochem. Pharmacol.* 19: 2461–68
93. Kato, R., Onoda, K., Takanaka, A. 1971. *Biochem. Pharmacol.* 20: 1093–1100
94. Solymoss, B., Classen, H. G., Varga, S. 1969. *Proc. Soc. Exp. Biol. Med.* 132:940–42
95. Stripp, B., Hamrick, M., Zampaglione, N. 1970. *Fed. Proc.* 29: 346
96. Feller, D. R., Gerald, M. C. 1971. *Proc. Soc. Exp. Biol. Med.* 136: 1347–50
97. Gerald, M. C., Feller, D. R. 1970. *Biochem. Pharmacol.* 19:2529–32
98. Gerald, M. C., Feller, D. R. 1970. *Arch. Int. Pharmacodyn.* 187: 120–24
99. Dingell, J. V., Heimberg, M. 1968. *Biochem. Pharmacol.* 17:1269–78
100. Neubert, D., Maibauer, D. 1959. *Arch. Exp. Pathol. Pharmakol.* 235:291–300
101. Smuckler, E. A., Arrhenuis, E., Hultin, T. 1967. *Biochem. J.* 103:55–64
102. Castro, J. A., Sasame, H. A., Sussman, H., Gillette, J. R. 1968. *Life Sci.* (Part I):129–36
103. Sasame, H. A., Castro, J. A., Gillette, J. R. 1968. *Biochem. Pharmacol.* 17:1759–68
104. Greene, F. E., Stripp, B., Gillette, J. R. 1969. *Biochem. Pharmacol.* 18:1531–3
105. Castro, J. A., Sasame, H. A., Greene, F. E., Gillette, J. R. Unpublished results
106. Marchand, C., McLean, S., Plaa, G. 1970. *J. Pharmacol. Exp. Ther.* 174:232–8
107. Ziegler, D. M., Jollow, D., Cook, D. E. 1970. *Flavins and Flavo-proteins: Int. Symp.*, ed. H. Kamin, University Park Press: Baltimore. 520 pp.
108. Machinist, J. M., Orme-Johnson, W. H., Ziegler, D. M. 1966. *Biochemistry* 5:2939–43
109. Ziegler, D. M., Pettit, F. H. 1966. *Biochemistry* 5:2932–38
110. Bickel, M. H. 1969. *Pharmacol. Rev.* 21:325–55
111. Masters, B. S. S. et al. 1971. *Chem. Biol. Interactions* 3:296–99
112. Ziegler, D. M., Mitchell, C. H., Jollow, D. See Ref. 4, 173–88
113. Boyland, E., Chasseaud, L. F. 1969. *Advan. Enzymol.* 32:173–219
114. Holtzman, J. L., Gillette, J. R., Milne, G. W. A. 1967. *J. Am. Chem. Soc.* 89:6341–44
115. Jerina, D. M., Daly, J. W., Witkop, B., Zaltzman-Nirenberg, P., Udenfriend, S. 1969. *Biochemistry* 9:147–56
116. Jerina, D. M., Daly, J. W., Witkop, B., Zaltzman-Nirenberg, P., Udenfriend, S. 1968. *Arch. Biochem. Biophys.* 128:176–83
117. Maynert, E. W., Foreman, R. L.,

Watabe, T. 1970. *J. Biol. Chem.* 20:5234–38
118. Leibman, K. C., Ortiz, E. 1968. *Pharmacologist* 10:203
119. Leibman, K. C., Ortiz, E. 1968. *Mol. Pharmacol.* 4:201–07
120. Zampaglione, N., Jollow, D., Hamrick, M., Stripp, B., Gillette, J. R. 1971. *Fed. Proc.* 30:448
121. Ames, B. N. 1971. *Chemical Mutagens: Principles and Methods for their Detection,* ed. A. Hollaender, 267–82. Plenum: New York
122. Boyland, E. 1964. *Brit. Med. Bull.* 20:121–26
123. Brodie, B. B. et al. 1971. *Proc. Nat. Acad. Sci.* 68:160–64
124. Hucker, H. B., Michniewicz, B. M., Rhodes, R. E. 1971. *Biochem. Pharmacol.* 20:2123–9
125. Parli, C. J., Wang, N., McMahon, R. E. 1971. *Biochem. Biophys. Res. Commun.* 43:1204–09
126. Smith, R. L., Dring, L. G. 1970. *Amphetamines and Related Compounds,* eds. E. Costa, S. Garattini, 121–40. Raven: New York 962 pp.
127. Gillette, J. R., Kamm, J. J., Sasame, H. A. 1968. *Mol. Pharmacol.* 4: 541–48
128. Kato, R., Oshima, T., Tokanaka, A. 1969. *Mol. Pharmacol.* 5:487–98
129. Fouts, J. R., Brodie, B. B. 1957. *J. Pharmacol. Exp. Ther.* 119:197–207
130. Morita, M., Feller, D. R., Gillette, J. R. 1971. *Biochem. Pharmacol.* 20:217–26
131. Feller, D. R., Morita, M., Gillette, J. R. 1971. *Biochem. Pharmacol.* 20:203–15
132. Kato, R., Takahashi, A. 1970. *Biochem. Pharmacol.* 19:45–55
133. Hernandez, P. H., Gillette, J. R., Mazel, P. 1967. *Biochem. Pharmacol.* 16:1859–76
134. Gillette, J. R. 1971. *Concepts in Biochemical Pharmacology: Handbook of Experimental Pharmacology,* eds. B. B. Brodie, J. R. Gillette, vol. 28, Part 2, Springer-Verlag: Berlin-Heidelberg-New York. 778 pp. In press
135. Shargell, L. D. 1969. *Influence of electron carrier systems in the microsomal metabolism of drugs.* Ph.D. thesis. George Washington University, Washington, D.C.
136. Watermann, M. R., Mason, H. R. 1970. *Biochem. Biophys. Res. Commun.* 39:450–54
137. McMahon, R. E. See Ref. 134
138. Moir, N. J., Miller, W. L., Gaylor, J. L. 1968. *Biochem. Biophys. Res. Commun.* 33:916–21
139. Carter, E. A., Isselbacher, K. J. 1971. *Ann. N. Y. Acad. Sci.* 179: 282–94
140. Gillette, J. R., Brodie, B. B., La Du, B. N. 1957. *J. Pharmacol. Exp. Ther.* 119:532–40
141. Leiber, C. S., De Carli, L. M. 1968. *Science* 162:917–18
142. Leiber, C. S., De Carli, L. M. 1971. *Chem. Biol. Interactions* 3:292–93
143. Gillette, J. R. 1971. *Ann. N. Y. Acad. Sci.* 179:43–66
144. Whitsett, T. L., Dayton, P. G., McNay, J. L. 1971. *J. Pharmacol. Exp. Ther.* 171:246–55
145. Marchand, C., Brodeur, J. 1970. *Rev. Can. Biol.* 29:293–98
146. Klaassen, C. D. 1970. *J. Pharmacol. Exp. Ther.* 175:289–300
147. Levy, G. 1971. *Ann. N. Y. Acad. Sci.* 179:32–42
148. De Baun, J. R., Smith, J. Y. R., Miller, E. C., Miller, J. A. 1970. *Science* 167:184–86
149. Wattenberg, L. W., Leong, J. L. 1962. *J. Histochem. Cytochem.* 10:412–20
150. Burger, P. C., Herdson, P. B. 1966. *Am. J. Pathol.* 48:793–809
151. Reid, W. D., Eichelbaum, M., Christie, B., Brodie, B. B. 1971. *Fed. Proc.* 30:439
152. Zampaglione, N. G., Jollow, D. J., Mitchell, J. R., Gillette, J. R. In preparation
153. Jollow, D. J., Zampaglione, N. G., Mitchell, J. R., Gillette, J. R. In preparation
154. Reid, W. D., Christie, B., Eichelbaum, M., Krishna, G. 1971. *Exp. Mol. Pathol.* In press
155. Jollow, D. J., Zampaglione, N. G., Stripp, B., Hamrick, M. E., Gillette, J. R. In preparation