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MICROSOMAL CONVERSION OF SKF 525-A AND SKF 8742-A

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Abstract—Metabolism of SKF 525-A* and of SKF 8742-A has been studied in hepatic microsomal preparations from untreated and phenobarbitone-induced rats and from swine. Attempts were made to distinguish between metabolism by flavoprotein-dependent amine oxidase and metabolism by the NADPH-dependent cytochrome P-450 system by measuring the effects of pH on binding spectra; and the effects of agents which inhibit cytochrome P-450 and of dithiothreitol, which inhibits functions of the amine oxidase. It would appear that the contribution to the metabolism of SKF 525-A by the amine oxidase is minimal.

It has long been known that SKF 525-A (2-diethylaminoethyl 2,2,diphenylvalerate hydrochloride) prolongs the action of a number of medicaments [1, 2]. It does so largely through inhibition of the metabolism of these agents [3], resulting in a lengthening of their biological half-lives [4]. Although SKF 525-A is an active inhibitor of a wide range of metabolic transformations of drugs by reactions such as side chain oxidation, *N*-dealkylation, *O*-demethylation and deamination [5–7], it has little or no effect on the hepatic microsomal metabolism of a number of other drugs [1, 6–12]. Variation in this regard exists from one species to another [9, 13], and induction of the hepatic enzymes with phenobarbitone or with 3-methylcholanthrene brings with it an alteration in the inhibitory pattern of SKF 525-A [14, 15]. There have been a number of proposals, and some debate, on the mechanism of the inhibition caused by SKF 525-A [3, 4, 16–23] but an adequate explanation of the biotransformation data has yet to be put forth. A partial explanation is found in the existence of competition between the drug and the inhibitor for binding site on the terminal electron acceptor of the hepatic mixed-function oxidase, the cytochrome P-450 [22], and the formation of a stable oxygenated complex of SKF 525-A, or a metabolite thereof, and the reduced form of cytochrome P-450 [23]. Such mechanisms would not, however, adequately account for differential inhibitory effects *vis-à-vis* chemically related compounds, or for variations caused by enzyme inducing agents, or for species differences. The possibility of an involvement of other microsomal enzymes, specifically the microsomal mixed-function amine oxidase [24], has not yet been investigated. The amine oxidase could be involved both in the conversion and in the inhibitory effects of SKF 525-A. Past findings on the size of the SKF 525-A molecule [25], its basicity [26] and the fact that it is a tertiary amine might indicate that the hepatic amine oxidase may well be involved in the metabolism of SKF 525-A.

To shed more light on the inhibitory action of SKF 525-A we have studied the conversion of this inhibitor and its main metabolite, the mono-deethylated metabolite SKF 8742-A, both in an attempt to learn more about the metabolism of these compounds and about the enzymes acting on and being inhibited by them.

MATERIALS AND METHODS

SKF 525-A and SKF 8742-A were the gift of Smith, Kline & French International Co.; isocitrate dehydrogenase was obtained from Boehringer-Mannheim, F.R.G. Wistar rats were obtained from Tick Research Unit, Rhodes University.

Induction of hepatic mixed function oxidase. Female rats weighing from 200 to 250 g were injected intraperitoneally with 80 mg/kg phenobarbitone for three days before being killed.

Preparation of microsomes. Rats were killed by cervical dislocation and their livers quickly removed and perfused with cold 0.9% NaCl solution. The method of Cinti *et al.* [27] using Ca (II) precipitation followed by spinning in an MSE 18 high-speed centrifuge was employed to separate the microsomal fraction. Swine livers were obtained fresh from an abattoir.

Incubation experiments. Incubations were carried out at 38° with shaking. The incubation mixture contained $MgCl_2$ (5 mM), $MnCl_2$ (5 μM), NADP (1.4 mg), tri sodium isocitrate (2.5 mg), SKF 525-A or SKF 8742-A (1.0 mg), microsomal suspension (0.6 mg protein in 1.0 ml), isocitrate dehydrogenase (0.2 mg), made up to a final volume of 2.0 ml with Tris–HCl buffer of pH 7.8 [28–30]. Dithiothreitol or *n*-octylamine, when used, was added before the microsomes and isocitrate dehydrogenase. The suspensions were gassed with O_2 and the flasks stoppered at the beginning of the incubation. The process was stopped after 30 min with 1 ml 10% trichloroacetic acid. Extent of metabolism was assessed as the difference between metabolism in the complete incubation mixture and that in the absence of NADPH generating system.

Determination of pH optimum. The influence of

* Abbreviations used: SKF 525-A, 2-diethylaminoethyl 2,2-diphenylvalerate hydrochloride; SKF 8742-A, 2-ethylaminoethyl 2,2-diphenylvalerate hydrochloride.

pH on the binding of SKF 525-A to microsomes was studied in buffers ranging in pH from 7.0 to 8.6 at intervals of 0.1 pH units. Binding was detected by means of difference spectra recorded on a Beckman Acta M VI Spectrophotometer. Metabolic studies were carried out in the incubation mixture as outlined with the exception of the buffers. Metabolism was measured as below.

Analysis of reaction products. The reaction products isolated by extraction with ether (3 × 3 ml) were analysed in a Hewlett Packard 5830 chromatograph fitted with 2 m × 3 mm O.D. stainless steel columns packed with DMCS-treated chromosorb G coated with 15% Apiezon L. Carrier gas: 60 p.s.i. high purity N_2, 30 p.s.i. FID hydrogen and 28 p.s.i. FID air. Retention time: SKF 8742-A: 8.97 min; principal minor metabolite: 7.31 min. Quantitation by area per cent method.

RESULTS

pH Optimum. The optimum pH for the binding of SKF 525-A to microsomes from untreated rats was 7.8. Metabolic studies on SKF 525-A, in which the metabolites were quantitated by g.c., showed that the greatest yield of SKF 8742-A occurred at pH 7.8 whereas that of metabolite I occurred at pH 7.6.

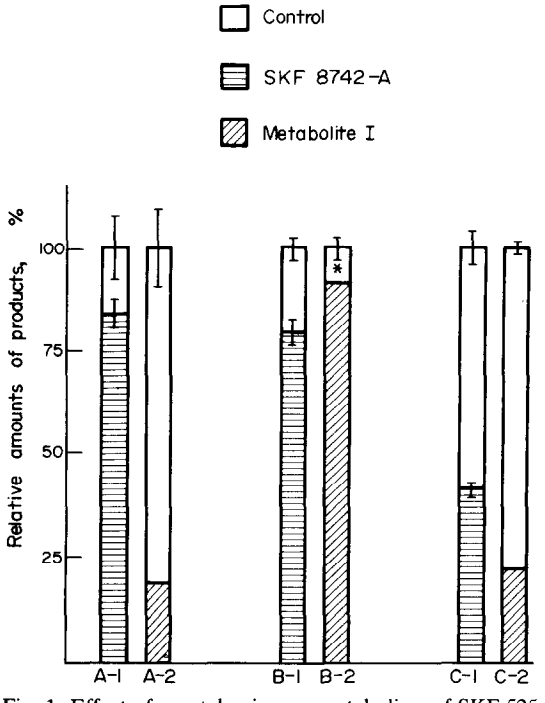

Fig. 1. Effect of *n*-octylamine on metabolism of SKF 525-A. Comparison of production of metabolites under two sets of conditions: with and without *n*-octylamine (10^{-3} M). A-1 and A-2 represent metabolism in the presence of untreated rat-liver microsomes; B-1 and B-2, phenobarbitone-treated rat-liver microsomes; and C-1 and C-2, uninduced swine-liver microsomes. 100 represents the amount of the metabolite in question formed in the absence of *n*-octylamine, whereas the hatched bars represent its formation in the presence of *n*-octylamine. *The metabolites I and IV peaks merged and were thus expressed as one value.

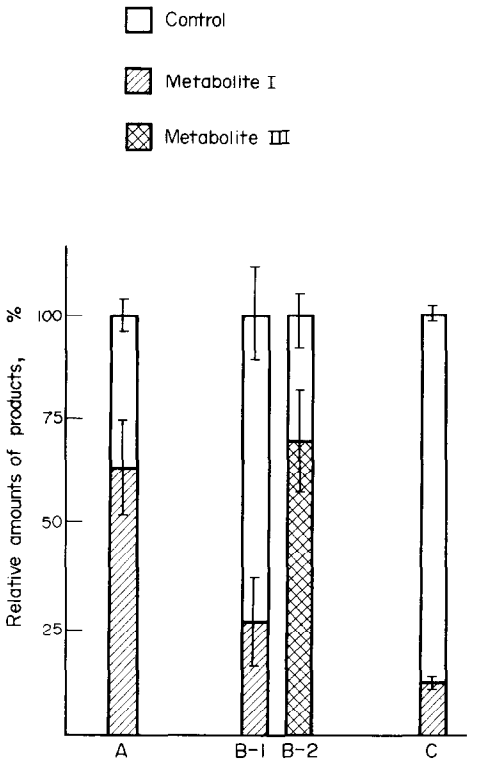

Fig. 2. Effect of *n*-octylamine on metabolism of SKF 8742-A. Comparison of production of metabolites under two sets of conditions: with and without *n*-octylamine (10^{-3} M). A represents metabolism in the presence of untreated rat-liver microsomes; B-1 and B-2, phenobarbitone-treated rat-liver microsomes; and C, swine-liver microsomes. 100 represents the amount of metabolite in question formed in the absence of *n*-octylamine whereas the hatched bars represent its formation in the presence of *n*-octylamine.

Metabolism of SKF 525-A. G.C. analysis of the incubation mixture of SKF 525-A and hepatic microsomes prepared from untreated rats showed the presence of SKF 8742-A, the mono-deethylated product, and a minor metabolite [31], metabolite I. When microsomes prepared from phenobarbitone-treated rats were used, production of SKF 8742-A almost doubled and the extract contained yet another metabolite, metabolite IV. Swine hepatic microsomes produced only SKF 8742-A and metabolite I under similar conditions.

Metabolism of SKF 8742-A. When SKF 8742-A was used as substrate, hepatic microsomes from untreated rats or from swine produced only metabolite I, but when phenobarbitone-pretreated rats were used, both metabolite I and metabolite III could be detected.

Effects of n-octylamine. *n*-Octylamine added to the incubation mixture of rat hepatic microsomes caused a slight reduction in the extent of conversion of SKF 525-A to SKF 8742-A but a marked reduction in the formation of metabolite I (Fig. 1 A-1, A-2). Under the same conditions, the presence of *n*-octylamine caused only slight reduction in the conversion of SKF 8742-A, metabolite I and metabolite IV (Fig. 1 B-1, B-2) when microsomes prepared from rats induced with phenobarbitone were used. In contrast

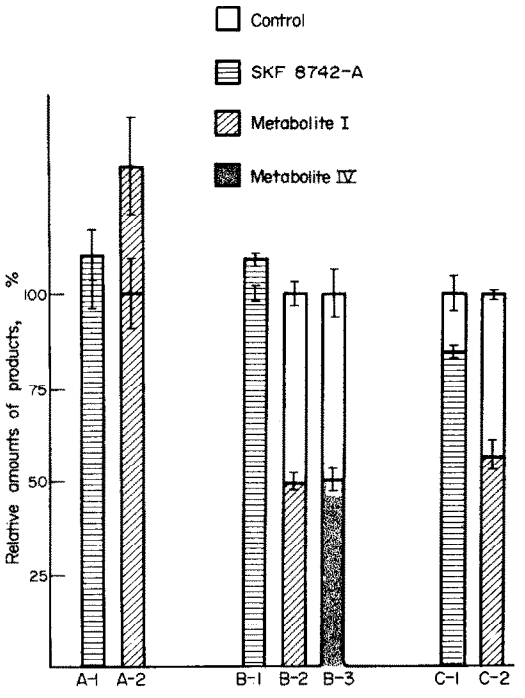

Fig. 3. Effect of dithiothreitol on metabolism of SKA 525-A. Comparison of production of metabolites under two sets of conditions: with and without dithiothreitol (1 mM). A-1 and A-2 represent metabolism in the presence of untreated rat-liver microsomes; B-1, B-2 and B-3, phenobarbitone pretreated rat-liver microsomes; and C-1 and C-2, untreated swine-liver microsomes. 100 represents the amount of metabolite in question formed in the absence of dithiothreitol whereas the hatched bars represent its formation in the presence of dithiothreitol.

to the finding reported by Poulsen *et al.* [32], when swine hepatic microsomes were used, we noticed a marked reduction in the formation of both SKF 8742-A and metabolite I (Fig. 1 C-1, C-2). In the presence of *n*-octylamine, formation of metabolite I from SKF 8742-A by microsomes from untreated rats (Fig. 2A), from phenobarbitone-induced rats (Fig. 2 B-1) as well as from swine hepatic microsomes (Fig. 2 C) was inhibited. Formation of metabolite III was also inhibited in the induced microsomal preparation (Fig. 2 B-2).

Effects of dithiothreitol. Addition of dithiothreitol to the incubation medium did not inhibit, or inhibited only slightly, the formation of SKF 8742-A from SKF 525-A by microsomes from either untreated (Fig. 3 A-1) or phenobarbitone-induced rats (Fig. 3 B-1). However, the same concentration of dithiothreitol (10^{-3} M) inhibited formation of metabolite I by about 50 per cent in the case of microsomes from phenobarbitone-induced rats (Fig. 3 B-2) and by about 40 per cent in the case of microsomal preparations from swine liver (Fig. 3 C-2). Formation of metabolite IV was inhibited to about the same extent (Fig. 3 B-3).

Dithiothreitol also inhibited the formation of metabolite I from SKF 8742-A by about 40 per cent by microsomes from untreated rats (Fig. 4A), and from swine (Fig. 4C), but inhibited the formation of both metabolite I (Fig. 4 B-1) and metabolite III (Fig. 4 B-2) only to a small extent when incubated with microsomes from phenobarbitone-pretreated rats.

Effects of carbon monoxide. Bubbling of the incubation mixture with CO resulted in a reduction of formation of metabolites of SKF 525-A (Fig. 5) and of SKF 8742-A (Fig. 6), regardless of the source of the microsomes.

DISCUSSION

The continued use of SKF 525-A as an experimental tool in the study of drug metabolism makes it important to understand its mechanism of action. Because most metabolic studies involve the use of whole animals, isolated cells or microsomes, all of which are highly complex mixtures of enzymes, it is of some importance to establish which enzymes are actually being affected by this inhibitor.

Being a tertiary amine, SKF 525-A might interact with two enzymes; and the same is true of its metabolites as they too may be amino compounds. The substrate specificities of the two microsomal enzyme systems most involved in the metabolism of amines, viz. the amine oxidase and the cytochrome P-450-dependent system, have not yet been rigidly defined but there is some evidence [31] that the amine oxidase metabolizes basic amines preferentially and that

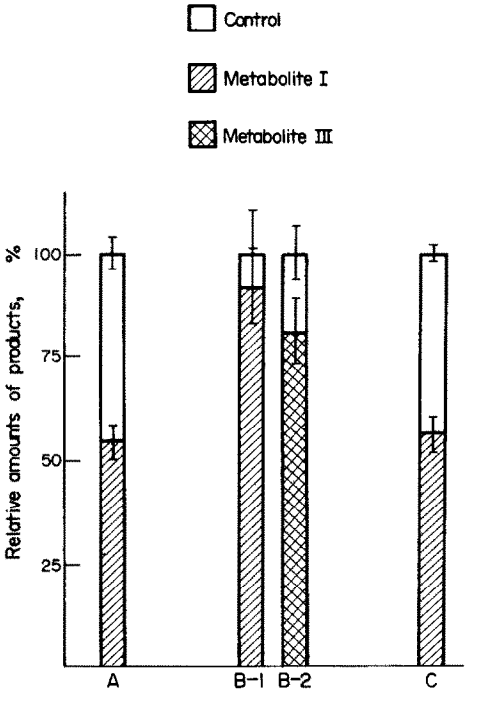

Fig. 4. Effect of dithiothreitol on metabolism of SKF 8742-A. Comparison of production of metabolites under two sets of conditions: with and without dithiothreitol (1 mM). A represents metabolism in the presence of untreated rat-liver microsomes; B-1 and B-2, phenobarbitone-treated rat-liver microsomes; and C, swine-liver microsomes. 100 represents the amount of metabolite in question formed in the absence of dithiothreitol whereas the hatched bars represent its formation in the presence of dithiothreitol.

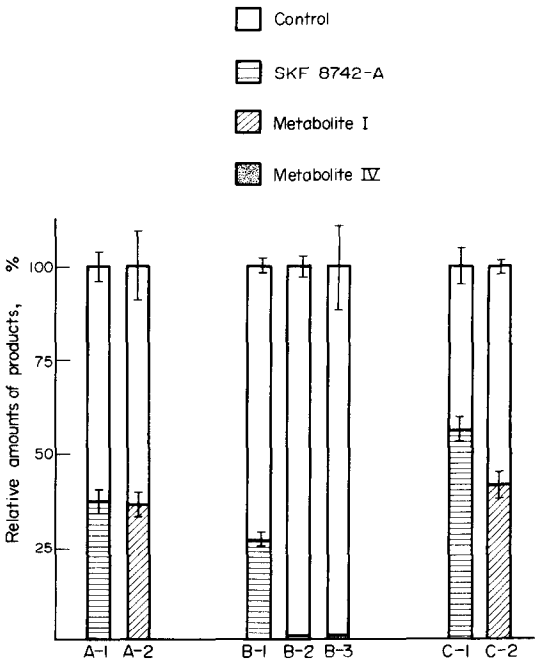

Fig. 5. Effect of carbon monoxide on metabolism of SKF 525-A. Comparison of production of metabolites under two sets of conditions: with and without CO. A-1 and A-2 represent metabolism in the presence of untreated rat-liver microsomes; B-1, B-2 and B-3, phenobarbitone-treated rat-liver microsomes; and C-1 and C-2, swine-liver microsomes. 100 represents the amount of metabolite in question formed in the absence of CO whereas the hatched bars represent its formation after microsomes had been bubbled for 2 min with a mixture of $CO:O_2:N_2$ (4:4:92, v/v).

the cytochrome system interacts more readily with weakly basic amines. It is therefore most likely that the cytochrome P-450 dependent system would interact more readily with SKF 525-A but that the metabolites may be further converted by either the cytochrome-dependent system or the amine oxidase.

The chemical nature of the metabolic products will usually distinguish between the two pathways dictated by the two enzymes, but isolation and identification of these metabolites is often difficult. In the case of SKF 525-A, only the major metabolite, SKF 8742-A, has been identified with certainty; on exposure to rat hepatic microsomal preparations SKF 525-A forms, in addition to the mono-deethylated product, metabolite I and, when exposed to microsomes from phenobarbitone-induced rats, metabolite IV as well. As the identity of metabolites I and IV has not yet been established, it is necessary to draw conclusions about the enzymatic involvement on the basis of other criteria.

If SKF 525-A were transformed solely by cytochrome P-450, it would be expected that the pH optimum for the conversion would lie near pH 8.4 [32]. The pH optima found for both the conversion of SKF 525-A and SKF 8742-A were closer to those expected for involvement of the cytochrome system. In attempting to draw a conclusion based on the measurement of metabolite levels, one must bear in mind, however, that various metabolites may not be equally stable at all pH values. For example, *N*-oxidases which are commonly formed when amine oxidase serves as catalyst [33, 34], are often quite unstable compounds and would be expected to decompose more rapidly at pH values above 8. If metabolite I is such a compound, more of it would survive and thus accumulate at lower pH levels where its stability is greater, even though its formation might be faster at higher pH values. Thus, were the amine oxidase involved, a pH study might give inconclusive results.

The amine oxidase has been shown to bring about the *N*-oxidation of some tertiary and secondary amines [33, 34]. This reaction is stimulated by *n*-octylamine, which, on the other hand, acts as an inhibitor of the reactions mediated by cytochrome P-450 [35, 36]. Our data show that *n*-octylamine added to rat or swine microsomes does not increase the rate of conversion of SKF 525-A (Fig. 1), nor of SKF 8742-A (Fig. 2). The evidence is thus against implication of the amine oxidase in the *N*-deethylation of SKF 525-A, in the formation of its two metabolites, I and IV, and in the formation of metabolite III from SKF 8742-A. In the rat microsomes, however, *n*-octylamine is not a potent inhibitor, a fact which may be explained by the high affinity of SKF 525-A for cytochrome P-450 [37].

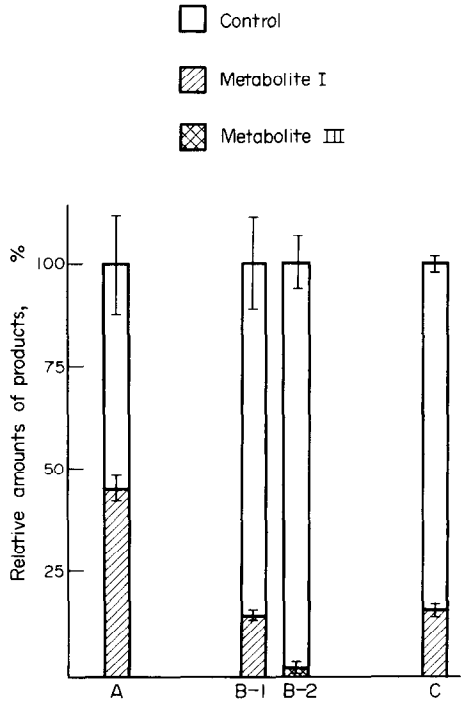

Fig. 6. Effect of carbon monoxide on metabolism of SKF 8742-A. Comparison of production of metabolites under two sets of conditions: with and without CO. A represents metabolism in the presence of untreated rat-liver microsomes; B-1 and B-2, phenobarbitone-treated rat-liver microsomes; and C, swine-liver microsomes. 100 represents the amount of metabolite in question formed in the absence of CO whereas the hatched bars represents its formation after microsomes had been bubbled for 2 min with a mixture of $CO:O_2:N_2$ (4:4:92, v/v).

Metabolite I arises when either SKF 525-A or SKF 8742-A is used as substrate. As compared with microsomes from untreated animals, microsomes from phenobarbitone-induced rats cause an increase in the formation of metabolite I from SKF 8742-A but only an insignificant increase in this metabolite when SKF 525-A serves as substrate. The evidence therefore indicates that the pathway runs from SKF 525-A to SKF 8742-A to metabolite I and favours the involvement of cytochrome P-450 in both conversions.

Stronger evidence for the involvement of cytochrome P-450 as the sole, or at least the major enzyme system, is provided by the observation that CO acts as a powerful inhibitor of the reaction *in vitro* of both SKF 525-A to its minor metabolites (Fig. 5) and SKF 8742-A to its metabolites (Fig. 6). What is more, NADPH and O_2 are obligatory cofactors for the reactions, as is shown by comparison with control incubations.

Had the amine oxidase been involved, one would also expect that dithiothreitol would slow down the reaction as this material itself serves as a substrate for the amine oxidase [32] and thus would provide substrate competition. But, on the contrary, dithiothreitol provides a slight stimulatory activity for the reaction with untreated rat liver microsomes (Fig. 3 A-1 and A-2), a stimulation which is probably the result of a protective effect of the dithiothreitol on the -SH group of the cytochrome. The difference in the results (Fig. 3 B-1 and B-2) obtained with microsomes from phenobarbitone-treated rats may be taken as an indication of the formation of different species of cytochrome P-450 in the presence of the inducing agent. In this case, whereas a similar increase in the formation of SKF 8742-A is noted by use of microsomes from pretreated rats, the increase in formation of metabolite I resulting when SKF 525-A served as the substrate in the presence of untreated rat liver microsomes does not occur when induced microsomes are used. A decrease is noted, as would be expected if amine oxidase were involved. It is consistent with the findings obtained when swine microsomes were used and when all three types of microsomes were allowed to react with SKF 8742-A (Fig. 4). These results favour the view that amine oxidase is indeed implicated in the conversion, in some cases at least.

All our experimental results support the conclusion that, in the case of untreated rats, the amine oxidase does not contribute measurably to the formation of the major or minor metabolites of SKF 525-A. Were this enzyme system involved, it would have been expected that the pH optimum would be near 8.4, that the reaction would have been stimulated by *n*-octylamine, and that carbon monoxide would have had little effect on the process. If *N*-oxide formations were involved, as might well be expected if the amine oxidase were involved, dithiothreitol would also have been expected to show clearer inhibitory effects. However, none of these presuppositions found support.

On the other hand, work with dithiothreitol tends to suggest that when microsomes from phenobarbitone-treated rats or from swine are used and when SKF 8742-A serves as the substrate there may be some involvement of the amine oxidase in the conversions.

It appears, therefore, that the differences in the inhibitory effects of SKF 525-A on metabolism of various xenobiotics and that differences found in various animal species might well be explained both on the basis of the presence of different species of cytochrome P-450 in different ratios and on the basis of the involvement of the amine oxidase under some circumstances. Where various species of cytochrome P-450 are present, each of these types then possesses its own substrate specificity and its unique binding affinity for SKF 525-A. Each will also behave differently toward other xenobiotics. The ratio of these individual types of cytochrome is likely to vary from one species of animal to another and with the environmental conditions to which the animal is subjected. Varying ratios of these types could explain the existence of some of the manifest differences between untreated and phenobarbitone-treated rats and differences noted between microsomes from swine and from treated and untreated rats.

Evidence for the involvement of amine oxidase in some conversions of SKF 525-A and SKF 8742-A would suggest that at least one of the minor metabolites might be an *N*-oxide [36, 38]. Studies are now under way to test this possibility.

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