ENHANCEMENT AND INHIBITION OF DRUG METABOLISM[1]

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A large number of factors can alter rates of drug metabolism. This review will discuss the enhancement and inhibition of drug metabolism. By enhancement is meant that increase in rate of in vitro drug metabolism observed following the inclusion of other drugs or chemicals in the reaction mixture. This term should be distinguished from the enzyme induction resulting from treatment of the intact animal with other drugs or hydrocarbons. Inhibition of drug metabolism, as used in this review, refers to the blockage of metabolism of one drug by another at the enzymic site. This restriction excludes those agents that reduce rates of drug metabolism by mechanisms other than competition at enzymic sites, such as treatment with carbon tetrachloride (1), morphine (2), thyroxine (3), and others. In addition, the review will be restricted to hepatic microsomal mixed function oxidases; enhancement and inhibition of hydrolytic, reductive, and conjugative pathways will not be considered.

ENHANCEMENT OF DRUG METABOLISM

Ethyl isocyanide.—Imai & Sato (4, 5) reported that EtNC exerts both stimulatory and inhibitory effects on microsomal aniline hydroxylation. The inhibitory effect of EtNC was attributed to a direct competition between oxygen and EtNC. The mechanism of the stimulatory effect appeared to be more complicated. The EtNC concentration producing maximal enhancement was a function of the aniline concentration; as the aniline concentration was increased, more EtNC was needed to produce maximal enhancement suggesting that both compounds were competing for the same enzyme system. In addition, species differences were observed in the activation of aniline hydroxylation by EtNC. The largest degree of enhancement was obtained in the rabbit; in contrast, EtNC produced only inhibition of aniline

[1] Abbreviations used in this review include: EtNC (ethyl isocyanide), SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate), SKF 8742-A (2-ethylaminoethyl 2,2-diphenylvalerate), SKF 26754-A (2-aminoethyl 2,2-diphenylvalerate), Lilly 18947 (2,4-dichloro-6-phenylphenoxyethyl-diethylamine), DPEA (2,4-dichloro-6-phenylphenoxyethylamine), K_m (Michaelis constant), V_{max} (maximal velocity), K_s (spectral binding constant) and K_i (inhibition constant).

metabolism when studied in guinea pig microsomal fractions, and results obtained with rat microsomal fractions were intermediate between those observed with rabbits and guinea pigs. The effect of pH on the action of EtNC on aniline hydroxylation was marked. It was observed that, at 3 mM EtNC, aniline hydroxylase activity was inhibited below pH 7.3 and progressively enhanced at pH values up to about 8.0. These findings may be related to the observation that reduced cytochrome P-450 exists in two forms which are in a pH dependent equilibrium (6). In an attempt to explain the stimulatory effect of EtNC on aniline hydroxylation, Imai & Sato proposed that the enhancement observed could be related to effects resulting from binding of EtNC to microsomal cytochromes. Specifically, previous studies by these authors had shown that EtNC binding increased the rate of P-450 reduction (7). In view of the fact that reduction of cytochrome P-450 is considered to be the rate limiting step in microsomal mixed function oxidations, it was suggested that EtNC exerted its enhancing effect by promoting the reduction of cytochrome P-450. In this mechanism it is proposed that both EtNC and aniline compete for oxidized cytochrome P-450. This serves to explain the observation that EtNC and aniline appear to compete for a common site in the enzyme system. Furthermore, it is suggested that the cytochrome P-450-EtNC complex is reduced at a faster rate than the cytochrome P-450-aniline complex. Following reduction of the cytochrome P-450-EtNC complex, oxygen or aniline displaces EtNC from cytochrome P-450 and the balance of the hydroxylation reaction takes place.

Acetone.—Anders (8) reported that acetone markedly enhanced the microsomal hydroxylation of aniline. Maximal enhancement of 200–300% occurred between acetone concentrations of 0.45–0.9 M acetone. Rat hepatic microsomal fractions showed the greatest sensitivity to the effects of acetone, with rabbits being intermediate in sensitivity and mice and dogs lowest. The enhancing effect of acetone appeared to be specific for the aromatic hydroxylase, since the hydroxylation of acetanilide and N-butylaniline was also increased by acetone; in contrast, no enhancement of the N-demethylation of ethylmorphine, N-methyl or N,N-dimethylaniline, nor the O-demethylation of *p*-nitroanisole was observed. The degree of enhancement increased with increasing pH in a manner similar to that reported with EtNC (4, 5). The kinetics of aniline hydroxylation were altered by acetone. In the presence of 0.045 and 0.45 M acetone, the K_m for aniline hydroxylation increased from a control value of 6.9×10^{-5} M to 13.0 and 19.9×10^{-5} M, respectively; similarly, the V_{max} increased from a control value of 1.4 μmoles/g liver /hr to 2.1 and 3.9 μmoles/g liver/hr, respectively. In addition, SKF 525-A and piperonyl butoxide proved to be more potent inhibitors of aniline metabolism in the presence of acetone. Acetone and EtNC produced approximately additive enhancements when included together in incubation mixtures containing rabbit liver microsomes.

While the mechanism by which acetone enhances microsomal aromatic

hydroxylation remains unknown, Anders (8) has suggested a number of possible interpretations. Schiff base formation, as a result of a reaction between acetone and aniline, was considered. However, the observation that the hydroxylation of acetanilide and N-butylaniline, compounds incapable of forming Schiff bases, was enhanced by acetone appeared to rule out Schiff base formation as a factor. The observation (4, 5) that EtNC enhances aniline hydroxylation suggested that EtNC and acetone might produce their effects by related mechanisms. Indeed, the effect of pH on the enhancements produced by EtNC and acetone was similar. However, a number of observed differences make a common mechanism unlikely. For example, aniline hydroxylation in the rat was markedly stimulated by acetone whereas EtNC produced only minimal enhancement in this species. In addition, the enhancing effect of EtNC was increased when pure oxygen rather than air was employed as the atmosphere in the incubation flasks; this change produced no effect on the enhancement due to acetone. Finally, it was suggested that if both acetone and EtNC act in a similar manner, it should not be possible to produce an additive enhancement with EtNC in the presence of maximally enhancing concentrations of acetone. Experimentally, it was observed that EtNC produced an enhancement even in the presence of high concentrations of acetone. These results strongly suggest that acetone and EtNC enhance microsomal hydroxylase activity by different mechanisms.

Kinetic studies suggested a possible explanation for the action of acetone (8). The finding that the K_m for aniline hydroxylation was altered in the presence of acetone would tend to rule out a simple increase in the amount of hydroxylase as a factor. It is possible, however, that acetone may unmask an aniline hydroxylase with different kinetic properties. Alternatively, two aniline hydroxylases differing in kinetic properties and in response to acetone, may be present in hepatic microsomes. Furthermore, one hydroxylase may be characterized by both a low K_m and V_{max} for aniline hydroxylation and a high susceptibility to inhibition by acetone; a second hydroxylase may, on the other hand, possess a high K_m and V_{max} and little susceptibility to inhibition by acetone. The former enzyme, because of its kinetic properties, would predominate in the absence of acetone. However, in the presence of acetone the increase in K_m and V_{max} would suggest that the latter hydroxylase was functioning, the former now being inhibited by acetone. This hypothesis also provides a possible explanation for the increased susceptibility to inhibition by SKF 525-A and piperonyl butoxide, since the two hydroxylases might differ with respect to the action of inhibitors. Support for the concept that two aromatic hydroxylases are present in hepatic microsomes has been offered by Daly et al (9). These workers observed that the migration and retention of deuterium during the hydroxylation of acetanilide-4-^{2}H was increased by previous treatment with phenobarbital and decreased following benzpyrene or 3-methylcholanthrene treatment; these results could be interpreted as being attributable to the presence of two

hydroxylases whose relative concentrations were altered by drug treatment. Wada et al (10) have also presented kinetic evidence suggesting the presence of two aniline hydroxylases in hepatic microsomes.

The enhancement of aromatic hydroxylation by acetone may reflect an increased rate of breakdown of an enzyme-activator-substrate complex as compared to the enzyme-substrate complex (11). Data was presented by Anders (8) that is consistent with this mechanism. Acetone did not alter the binding spectrum (9) of aniline to rat hepatic microsomes (M. W. Anders, unpublished observations). It should be noted that acetone itself failed to exhibit a binding spectrum with rat microsomes (13) but did bind to rabbit microsomes at high concentrations (14). Therefore, if it is assumed that binding spectra reflect the interaction of aniline with the active site of the enzyme, it follows that acetone may be acting at another site in the pathway, possibly by enhancing the dissociation of the enzyme-substrate complex. The finding that acetone acted as an uncompetitive activator as shown by the effects on the kinetics of the reaction (K_m/V_{max} is constant) is in agreement with this type of mechanism.

The effect of acetone on the migration and retention of deuterium in acetanilide-4-^{2}H hydroxylation has been reported (9). It was observed that acetone caused an increased migration and retention of deuterium in microsomes from rats treated with phenobarbital or 3-methylcholanthrene and in control and phenobarbital treated rabbits. It was also noted that acetone enhanced the hydroxylation of anisole-4-^{2}H and 4-fluorobiphenyl-4-^{2}H but had no effect on the migration and retention of the label in these compounds.

2,2′-Bipyridine.—Anders (15) reported that 2,2′-bipyridine enhanced the hydroxylation of aniline by microsomal fractions. In contrast to acetone, 2,2′-bipyridine also stimulated the N-dealkylation of N-methyl and N,N-dimethylaniline but inhibited the N-demethylation of ethylmorphine and aminopyrine. The enhancement due to 2,2′-bipyridine appeared not to be related to an inhibition of microsomal lipid peroxidation with a concomitant increase in aromatic hydroxylation. Inhibitors of lipid peroxidation have been shown to stimulate the epoxidation of aldrin to dieldrin (16). Although 2,2′-bipyridine altered the kinetics of aniline hydroxylation, the effect was not as marked as in the case of acetone. Studies of the effect of acetone on the enhancement due to 2,2′-bipyridine showed that the agents did not produce an additive stimulation. This could be interpreted as evidence that both compounds stimulate aromatic hydroxylation by the same mechanism. On the other hand, acetone may partially block the enhancing effect of 2,2′-bipyridine. A choice between these two alternatives could not be made on the basis of the available data.

Metyrapone.—Leibman (17) reported that metyrapone [SU-4885; 2-methyl-1,2-*bis*-(3-pyridyl)-1-propanone], an inhibitor of steroid 11β hydroxyla-

tion, inhibited the metabolism of hexobarbital and aminopyrine but enhanced the microsomal hydroxylation of acetanilide and trichloroethylene. Metyrapone enhanced acetanilide hydroxylation in both normal and phenobarbital treated rats; in contrast, only inhibition of trichloroethylene oxidation was seen in phenobarbital induced rats. Evidence was presented showing that metyrapone produced a reversible enhancement of trichloroethylene metabolism.

The mechanism by which metyrapone exerts its effects was studied by Hildebrandt, Leibman & Estabrook (18). These workers reported that metyrapone shows a type II binding spectrum (9) and that this compound is also bound to reduced cytochrome P-450. Furthermore, it was observed that two forms (P-446, P-454) of cytochrome P-450 are present in hepatic microsomes from rats. These could be detected by an effect of metyrapone on the enzymatic reduction of cytochrome P-450 and by the reaction of reduced cytochrome P-450 with oxygen or carbon monoxide. It was demonstrated that metyrapone enhanced the reduction of one form of cytochrome P-450 while exerting an inhibitory effect on the reduction of the second form. In addition, metyrapone inhibited the oxidation of reduced cytochrome P-450. These authors suggest that P-446 is involved in the metabolism of type I compounds (i.e., hexobarbital) and that P-454 participates in the hydroxylation of type II compounds (i.e., aniline, acetanilide). It is further postulated that the enhancing effect of metyrapone is due either to the formation of an inactive P-446-metyrapone complex, which would shunt electrons to P-454, or to a shift in the equilibrium between P-446 and P-454 to P-454. The former effect would explain the inhibition of hexobarbital metabolism by metyrapone while the latter would functionally increase the amount of enzyme available for acetanilide hydroxylation.

The effects of metyrapone on cytochrome P-450 reduction (18) are of particular interest in view of other studies dealing with cytochrome P-450 reductase. Gigon, Gram & Gillette (19) reported that type I substrates stimulate cytochrome P-450 reduction while type II substrates inhibit this reaction. Both aniline and acetanilide produce type II binding spectra (9). It is possible, therefore, that the mechanism of enhancement is related to cytochrome P-450 reduction. The stimulation observed may be the result of a reversal of the intrinsic inhibition of cytochrome P-450 reduction by type II substrates.

Inhibition of Drug Metabolism

Interest in the inhibition of microsomal drug metabolism stems from the reports of Cook and coworkers on the effects of SKF 525-A on the action of a number of drugs. It was observed (20) that SKF 525-A prolonged the hypnotic action of hexobarbital in rats and mice. The toxicity of hexobarbital was not significantly altered by SKF 525-A. Furthermore, it was reported that SKF 525-A was effective when administered orally or intraperitoneally. The prolongation of hexobarbital hypnosis began immediately after

administration of SKF 525-A and persisted for about 15 hr. The prolonging effect of SKF 525-A was dose related.

These results were extended (21) to include the effect of SKF 525-A on a number of CNS depressants. It was observed that SKF 525-A prolonged the hypnotic action of secobarbital, pentobarbital, hexobarbital, amobarbital, butethal, ortal, phenobarbital, and chloral hydrate. In contrast, no effect of SKF 525-A on the hypnosis due to barbital, thioethamyl, thiopental, and methylparafynol was detected. Additional studies (22) showed that SKF 525-A enhanced the analgesic action of methadone, meperidine, morphine, codeine, and methorphinan. The toxicity of the analgesics was not increased by SKF 525-A.

Studies by Brodie and coworkers (23) showed that SKF 525-A prolonged barbiturate hypnosis by inhibiting the in vivo biotransformation of the compound. In addition, blood levels of aminopyrine, ephedrine, and meperidine were prolonged in SKF 525-A treated animals. The effects of SKF 525-A were also studied at the in vitro level (24). It was shown that SKF 525-A inhibited enzyme systems located in liver slices which metabolize pentobarbital, secobarbital, hexobarbital, meperidine, dibenamine, aminopyrine, amphetamine, and codeine. In addition, the conjugation of morphine with glucuronic acid was inhibited.

These reports were followed by others describing additional inhibitors of drug metabolism. Fouts & Brodie reported the inhibition of drug metabolism by Lilly 18947 (25) and iproniazid (26). These compounds both appeared to act in a manner similar to SKF 525-A. Neubert & Herken (27) reported the inhibition of drug metabolism by various substituted phenylacetic acid esters of 2-diethylaminoethanol, and Kramer & Arrigoni-Martelli (28) studied the effects of malonic acid derivatives on hexobarbital sleeping time and metabolism. McMahon & Mills (29) showed that DPEA, the primary amine analogue of Lilly 18947, was a competitive inhibitor of N-demethylation.

In vitro studies.—The mechanism by which SKF 525-A and other inhibitors of drug metabolism exert their effect has been studied by a number of authors. Brodie (30, 31) has suggested two possibilities for the action of SKF 525-A: (*a*) inhibition of a common component of the microsomal enzyme systems, and (*b*) a physicochemical effect on the microsomal membrane caused by its interaction with the membrane to change its permeability to drugs. It is difficult to explain how a variety of reactions such as oxidation, reduction, hydrolysis, and conjugation could utilize the same microsomal component. Although TPNH is required by both the oxidative and reductive microsomal pathways, SKF 525-A has been shown to be without effect on TPNH oxidation by microsomes (32) or on TPN requiring enzymes such as G-6-P dehydrogenase, TPN-cytochrome c reductase, and cytochrome oxidase (24). Thus it would appear that if a common component is involved it is probably not TPNH. The contention that SKF 525-A may

prolong drug action by preventing the entry of drugs into the microsomes is also difficult to explain. It is known that SKF 525-A does not inhibit the dealkylation of either N-methyl or N-ethylaniline, compounds metabolized by the microsomal enzymes (33), yet SKF 525-A is a potent inhibitor of the N-demethylation of meperidine, also metabolized by the microsomes. Brodie (31) regards the inhibitory action of 2,2-diphenylvaleric acid, a hydrolysis product of SKF 525-A, as evidence in favor of the membrane hypothesis. Since this polar compound may not enter the microsome, it could be acting on the membrane. Although it is known that SKF 525-A possesses surface-active properties, the relationship of this property to its capacity to inhibit microsomal drug metabolism is unclear (34, 35).

Netter (36) has formulated a hypothesis to explain the mechanism of action of SKF 525-A. By analogy to the action of uncouplers of oxidative phosphorylation, it was suggested that perhaps SKF 525-A acts similarly on the drug metabolizing enzymes. It is supposed that the product of the TPNH oxidase reaction is an active peroxide or an active hydroxyl and that the actual drug oxidizing step is coupled to the oxidation of TPNH. SKF 525-A would then act in some way to uncouple the oxidation of TPNH from the drug oxidizing step. When no drug is present, the active hydroxyl might be destroyed by catalase or perhaps no active hydroxyl is formed in the absence of a drug substrate. In order to account for the failure of SKF 525-A to inhibit certain drug metabolizing reactions, one would have to postulate that a different reaction mechanism exists in those cases. Evidence has been presented purporting to show that SKF 525-A can dissociate TPNH oxidation and oxygen uptake in liver microsomes (37).

An early clue to the mechanism of action of inhibitors was provided by McMahon (38) who studied the nature of inhibition of microsomal demethylation by DPEA. It was observed that DPEA was a competitive inhibitor of the N-demethylation of butynamine and meperidine. In addition, DPEA inhibited the demethylation of a variety of substrates. SKF 525-A, 5-[α-aminopropylidene]-dibenzo[a,d]cyclohepta[1,4]diene, Lilly 18947, and demethylmeperidine were also shown to be competitive inhibitors of butynamine demethylation. From these studies McMahon (38) concluded that the substrate and inhibitor compete for the same site on the enzyme.

Rubin, Tephly & Mannering (39) postulated that if the microsomal enzyme system is nonspecific so that it will catalyze the oxidation of many drugs, then one drug should competitively inhibit the metabolism of another. Evidence was presented to support this view. Specifically it was shown that hexobarbital, chlorpromazine, zoxazolamine, phenylbutazone, and acetanilide were competitive inhibitors of the N-demethylation of ethylmorphine. These inhibitors are all known substrates for the microsomal enzymes. Barbital and acetazoleamide, compounds that are not significantly metabolized, did not inhibit ethylmorphine N-demethylation. In addition, it was observed that ethylmorphine, hexobarbital, and chlorpromazine were mutually inhibitory, each retarding the metabolism of the other. These results were interpreted

as evidence for a common enzyme pathway that accepts many drugs as substrates. This then suggested that one drug inhibited the metabolism of another by serving as an alternative substrate. There are three requirements for an alternative substrate mechanism of inhibition: (*a*) inhibition must be competitive, (*b*) the inhibitor must also serve as a substrate for the enzyme and (*c*) the K_m of the inhibitor when serving as a substrate must equal the K_i when serving as an inhibitor. It was observed that, in the cases of hexobarbital and ethylmorphine, these requirements were met. The K_i and K_m of chlorpromazine, however, were different, suggesting that the mechanism for this compound may be more complicated.

The studies cited above suggested a possible mechanism for the inhibition of drug metabolism by SKF 525-A, namely that this compound acts as an alternative substrate (40). This hypothesis required that SKF 525-A serve as a substrate in the microsomal enzymes. Indeed, an examination of the structure of SKF 525-A suggested numerous pathways for metabolism: N-dealkylation, aromatic hydroxylation, penultimate and terminal oxidation of the propyl side chain, and hydrolysis. To test the alternative substrate mechanism of inhibition, the kinetics of the N-deethylation of eleven known inhibitors were studied. All compounds were found to undergo N-dealkylation and all proved to be competitive inhibitors of the N-demethylation of ethylmorphine. Additional support for the alternative substrate mechanism was obtained from kinetic studies. For six of the compounds studied, the K_m and K_i were not significantly different. For most compounds whose K_m and K_i were different (including SKF 525-A), the K_i proved to be lower than expected and, in the case of SKF 525-A, the K_i decreased with increasing incubation time, indicating a more complicated mechanism. These results suggested that an alternative substrate mechanism could explain the action of a number of known inhibitors of drug metabolism. For other compounds, however, the alternative substrate mechanism provided an inadequate explanation of their action.

It should be noted that while a compound does not necessarily have to undergo metabolism to inhibit the microsomal enzymes, it must at least bind to the active site of the enzyme. An example is seen in the primary amines, DPEA (29) and SKF 26754-A (41), which presumably are not metabolized but still serve as potent inhibitors of drug metabolism. It should be pointed out, however, that both DPEA and SKF 26754-A could undergo deamination which can be regarded as a form of N-dealkylation.

A possible explanation for the failure of the K_m and K_i of SKF 525-A to agree with predictions is found in recent work by Mannering (42). These studies were based on the observation that SKF 525-A is firmly bound (41, 43) to microsomes as a type I compound (12). In these experiments, SKF 525-A was mixed with washed microsomes, the microsomes resedimented, washed, and examined for type I and II binding sites, SKF 525-A content, and ability to metabolize hexobarbital (type I) and aniline (type II). The

presence of a type I binding site could not be detected after the addition of hexobarbital or SKF 525-A to the microsomes although the type II binding site was still present after the addition of aniline. Dialysis reduced the SKF 525-A content to about 40 μmoles/μmole cytochrome P-450 but did not restore the type I site. The SKF 525-A treated microsomes metabolized ethylmorphine, hexobarbital, and aniline at 63, 31, and 67 percent of control microsomes, respectively. It was concluded that SKF 525-A binds irreversibly to the type I site but not the type II site, that it is bound to materials other than cytochrome P-450, and that metabolism of ethylmorphine and hexobarbital is possible without type I binding. These findings could explain the observed difference between the K_m and K_i of SKF 525-A, since this compound may be acting as an alternative substrate in addition to obliterating the type I site which would result in a lower K_i. Furthermore, the change in the K_i of SKF 525-A with incubation time may reflect the time course of the loss of the type I site.

Competitive inhibition has been frequently observed when the effect of one drug on the metabolism of another is studied. Brooks & Harrison (44) showed that the hydroxylation of dihydroaldrin was competitively inhibited by dieldrin and α-hexachlorohexane and that the epoxidation of aldrin was similarly inhibited by dihydroaldrin and α-hexachlorohexane. The O-demethylation of p-nitroanisole was competitively inhibited by disulfiram and diethyldithiocarbamate (45). Quinine and quinidine served as competitive inhibitors of pentobarbital metabolism (46). Estradiol-17β, testosterone, androsterone, progesterone, and hydrocortisone competitively inhibited the metabolism of ethylmorphine and hexobarbital (47); similar results were obtained with prednisolone and hydrocortisone as inhibitors of aminopyrine and aniline metabolism (10). Competitive inhibition of the epoxidation of heptachlor and aldrin was observed with dieldrin and heptachlor epoxide, respectively, serving as inhibitors (48). Insecticide synergists competitively inhibited naphthalene metabolism in housefly microsomes (49) and ethylmorphine in rat liver microsomes (50). Both metyrapone and reduced metyrapone were found to be competitive inhibitors of the demethylation of p-nitroanisole and N-methyl-p-nitroaniline (51).

It is tempting to speculate that an alternative substrate mechanism would suffice to explain most cases of inhibition of drug metabolism. However, one must reckon with the many reports describing noncompetitive or mixed kinetics that are incompatible with a simple alternative substrate mechanism.

In the case of SKF 525-A, noncompetitive inhibition has been observed with monomethyl-4-aminoantipyrine (52) or o-nitroanisole (53) as substrates. Similar kinetics were obtained when SKF 525-A served as an inhibitor of the O-demethylation of p-nitroanisole in microsomes derived from normal as well as phenobarbital or 3,4-benzpyrene treated rats (54). Chloramphenicol was shown to be a noncompetitive and irreversible inhibitor of

hexobarbital and acetanilide hydroxylation (55); isonicotinic acid hydrazide and *p*-amino salicylic acid produced noncompetitive inhibition of diphenylhydantoin hydroxylation (56).

The type of inhibition obtained is influenced by a number of factors including the substrate. For example, Schenkman, Ball & Estabrook (57) observed that nicotinamide was a competitive inhibitor of aminopyrine demethylation and a mixed inhibitor of aniline hydroxylation. These studies were important because nicotinamide has been routinely included in incubation mixtures (25, 26) to prevent the destruction of cofactors by pyridine nucleotidase (58).

Sasame & Gillette (59) have reported the results of an extensive study of the effects of various factors on the inhibition of drug metabolism. Specifically the inhibitory effects of type II compounds on the metabolism of type I substrates were studied. It was observed that nicotinamide was a competitive inhibitor of aminopyrine demethylation and a mixed inhibitor of ethylmorphine demethylation when rat microsomes were employed; inhibition studies with aniline as the substrate showed curved lines (cf. 57). In the mouse, however, different results were obtained in that nicotinamide proved to be a competitive inhibitor of aniline hydroxylation and a mixed inhibitor of aminopyrine demethylation; inhibition of ethylmorphine demethylation was noncompetitive at low nicotinamide levels although a different pattern was obtained at higher inhibitor concentrations. SKF 26754-A and DPEA proved to be uncompetitive inhibitors of aniline hydroxylation in mouse liver microsomes although DPEA produced additional effects. Different results were obtained when ethylmorphine served as the substrate. Using mouse liver microsomes it was observed that SKF 26754-A produced noncompetitive inhibition at low concentrations while both competitive and noncompetitive kinetics were obtained at higher levels of inhibitor. DPEA produced primarily noncompetitive inhibition of ethylmorphine N-demethylation.

The effect of nicotinamide on the inhibition of drug metabolism by SKF 26754-A and DPEA was also studied (59). It was observed that, in mouse liver microsomes, nicotinamide was a competitive inhibitor of aniline hydroxylation and both SKF 26754-A and DPEA produced uncompetitive inhibition. In the presence of nicotinamide, however, SKF 26754-A produced partially noncompetitive inhibition of aniline hydroxylation and competitive inhibition of ethylmorphine N-demethylation while DPEA produced both noncompetitive and competitive inhibition depending on the concentration of inhibitor. It was also observed that SKF 26754-A and DPEA increased the apparent K_s for ethylmorphine. SKF 26754-A acted in a competitive manner while DPEA produced both slope and intercept alterations.

The interaction of inhibitors with cytochrome P-450 reductase was also examined (59). Since type I and type II substrates had been observed to stimulate and depress cytochrome P-450 reduction (19), respectively, inhibitors could exert their effects at this point. It was noted that nicotinamide non-

competitively inhibited the stimulation of cytochrome P-450 reduction due to ethylmorphine while SKF 26754-A showed mixed inhibition. These effects were similar to those obtained when ethylmorphine demethylation was studied.

Sasame & Gillette (59) have concluded that since type II compounds inhibit the metabolism of type I substrates the interaction between the two binding sites must be complex. That this is indeed the case is shown by the variations in inhibition patterns obtained both with different substrates and species of experimental animals. The finding that the type of inhibition varied with the concentration of inhibitor, when ethylmorphine served as the substrate, was interpreted as evidence for the presence of two enzymes that metabolize ethylmorphine. It is of interest, at this point, to recall the studies of Mannering (42) cited above showing that ethylmorphine can be metabolized even though type I binding cannot be detected. Thus it is possible that the native enzyme and that remaining after the loss of the type I site could respond differently to the effects of inhibitors.

Finally, it was suggested (59) that type II compounds inhibit the metabolism of type I substrates both by slowing the reduction of cytochrome P-450 and by altering the affinity of type I binding sites to the substrate. That these effects are concentration dependent may reflect the presence of at least two type II binding sites and possibly two ethylmorphine metabolic sites.

Leibman, Hildebrandt & Estabrook (60) have studied the interactions between substrates in their binding to cytochrome P-450. They observed that aniline, a type II compound, produced a competitive inhibition of the binding spectrum of hexobarbital, a type I substrate. On the other hand, when both substrate and inhibitor were type II compounds, inhibition of binding was never found to be competitive. For example, metyrapone was found to be a noncompetitive inhibitor of aniline binding. The results obtained when both inhibitor and substrate were type I compounds varied. A competitive relationship was observed between hexobarbital and amobarbital but structurally unrelated compounds showed noncompetitive inhibition. Different results were obtained when the effect of type I compounds on the binding of type II compounds was studied. In this case the binding of the type II substrate was enhanced by the type I compound.

These authors (60) suggest that microsomes may contain two forms of oxidized hemoprotein, namely E and EX. The former exists uncombined with any substrate while the latter may represent a complex with endogenous materials or a membrane constituent. It is also supposed that E can react with type II compounds to form EII which shows the typical type II binding spectrum. Furthermore, reaction of EX with type I compounds could lead to the appearance of EI showing typical type I spectra. EI and EII are pictured as being interconvertible. Thus, reaction of EI with a type II substrate results in conversion to EII, which could explain the enhancement of type II binding by type I compounds. That the opposite result was

not obtained, namely that type II compounds did not enhance the binding of type I substrates, could be explained by the formation of an unreactive complex in this case.

Complex patterns of inhibition have been observed by other authors. Wada et al (10) found that the inhibition of aniline hydroxylation by steroids resulted in hyperbolic double reciprocal plots. At high substrate concentrations, inhibition by prednisolone appeared to be competitive. However, at lower aniline concentrations the patterns changed indicating a more complex form of inhibition. Inhibition by prednisolone was more pronounced in rats or mice treated with phenobarbital. In addition, prednisolone was shown to give a type II binding spectrum and to produce a diminution of the aniline binding spectrum. The kinetic results were interpreted as being suggestive of the presence of two aniline hydroxylases in liver microsomes. In view of the above results, it should be noted that Tephly & Mannering
were competitive inhibitors of ethylmorphine and hexobarbital metabolism. Similarly, Juchau & Fouts (61) reported that norethynodrel and progesterone were competitive inhibitors of hexobarbital metabolism. The latter workers (47, 61) used type I substrates whereas Wada et al (10) employed aniline, a type II compound, as a substrate; this could explain the differences in the results obtained.

Graham, Hellyer & Ryan (62) also noted curved double reciprocal plots while studying the inhibition of drug metabolism by naturally occurring compounds. Curved lines were obtained when piperonyl butoxide or grandiflorone served as inhibitors of aniline hydroxylation. Similar results were obtained with aminopyrine as the substrate. At high substrate concentrations competition appeared to be of an inhibition type; however, at lower substrate concentrations the degree of inhibition declined. One compound studied, leptospermone, produced only typical competitive inhibition at all substrate levels. The authors also studied the effect of preincubating the inhibitor with microsomes prior to addition of substrate. It was observed that the inhibition of aniline hydroxylation by grandiflorone was abolished by this treatment. Similar results were obtained with piperonyl butoxide as the inhibitor and aminopyrine as the substrate; preincubation of leptospermone did not alter its inhibition of aminopyrine demethylation.

These authors (62) attributed the curvature of the double reciprocal plots to metabolism of the inhibitor during the course of the incubation. They pointed out that more free enzyme would be available at lower substrate concentrations if excessive substrate is not present. Under these conditions, more enzyme would be free to metabolize and inactivate the inhibitor. The result would be a fall in inhibition with decreasing substrate concentration. Evidence for this view is the finding that leptospermone, a compound stable to incubation, produced only linear competitive inhibition.

Curved double reciprocal plots were noted in studies of aldrin epoxidation in housefly (63) and pig (16) microsomes when methylenedioxyben-

zenes served as inhibitors. This was tentatively interpreted (16) as being attributable to the presence of two distinct enzymes, both of which metabolize a common substrate. Furthermore, since no curvature was noted in the absence of inhibitors it was felt that the two enzymes must possess similar K_m; the curved double reciprocal plots suggested, though, that the two enzymes differed in their susceptibility to inhibitors. However, Casida et al (64) have shown that methylenedioxybenzenes do serve as substrates for microsomal enzymes, suggesting that metabolism of the inhibitor could have played a role in the aldrin epoxidation studies.

In vivo studies.—Early reports showed that SKF 525-A inhibited drug metabolism in vivo (23) as well as in vitro (24). Similar results showing the effectiveness of SKF 525-A and other compounds as in vivo inhibitors of drug metabolism have been reported. Anders (50) and Furner et al (65) have shown that methylenedioxybenzenes and SKF 525-A, respectively, increase the biologic half-life of hexobarbital in the rat. Boulos, Short & Davis (46) reported that quinine prolonged the half-life of pentobarbital in goats. Imipramine demethylation in the intact rat was inhibited by SKF 525-A (66). Furthermore, it has been shown that imipramine also inhibits the in vivo metabolism of carisoprodol, meprobamate, hexobarbital, or pentobarbital and potentiates the inhibitory effects of SKF 525-A (67). Chlorcyclizine, glutethimide, and phenaglycodol, along with SKF 525-A and DPEA, were found to inhibit hexobarbital, pentobarbital, meprobamate, and carisoprodol metabolism in rats (68). Other studies have demonstrated the inhibitory effects of SKF 525-A on the in vivo metabolism of mephenesin in dogs (69). McMahon et al (70) have studied the inhibitory effects of a series of structural analogs of DPEA. Desipramine has been shown to inhibit the metabolism of amphetamine in the intact rat (71), an observation confirmed by further studies using the isolated perfused rat liver (72). Disulfiram was found to prolong aminopyrine blood levels in rats (45).

The above reports show that many inhibitors of microsomal drug metabolism are effective both in vitro and in vivo. However, it is sometimes observed that certain compounds fail to inhibit drug metabolism significantly in the intact animal although inhibition may have been observed at the in vitro level. Thus, Rubin, Tephly & Mannering (73) observed that while ethylmorphine and codeine inhibited hexobarbital metabolism in the intact rat, morphine, norcodeine, dextromethorphan, levomethorphan, meprobamate, and acetanilide produced no inhibitory effect. These authors (73), as well as McMahon (38), have summarized the requirements for a compound to serve as an effective inhibitor of drug metabolism in vivo. These include: (*a*) absorption and distribution of the inhibitor must favor its accumulation in the liver, (*b*) the inhibitor must not produce toxic effects before inhibitory levels are reached in the liver, (*c*) the K_m of the inhibitor should not appreciably exceed that of the substrate, (*d*) the V_{max} of the inhibitor should not exceed that of the substrate so as to maintain its presence at the meta-

bolic site, (*e*) alternative pathways for metabolism (i.e., nonmicrosomal) should play a minor role in the disposition of the inhibitor, and (*f*) the inhibitor should not be excreted more rapidly than the substrate.

Renal excretion or excessive CNS depression or stimulation, for example, may be factors in failure of some inhibitors to be effective at the in vivo level. These problems can be avoided by use of the isolated perfused rat liver in studying drug metabolism as demonstrated by Stitzel, Anders & Mannering (74). Stitzel, Tephly & Mannering (75) studied the inhibition of hexobarbital metabolism in the perfused liver. Using this technique, morphine, levomethorphan, and dextromethorphan, compounds failing to produce inhibition in the intact rat (73), were found to inhibit significantly hexobarbital metabolism. Dingell & Bass (72) showed that desipramine inhibited amphetamine metabolism in the isolated perfused rat liver.

Factors affecting inhibition of drug metabolism.—Species differences have been demonstrated in the action of inhibitors of drug metabolism. McMahon (76) has studied the effect of SKF 525-A on the dealkylation of secondary and tertiary amines in several species. It was found that SKF 525-A (0.15 mM) produced 6, 51, and 0 percent inhibition of ephedrine demethylation in the rabbit, rat, and guinea-pig, respectively.

Kato, Onoda & Takayanagi (77) have studied species differences in the inhibition of drug metabolism by SKF 525-A, DPEA, chlorcyclizine, and U-16392-A (*o*-chloroisopropyl phenylhydrazine). It was observed that SKF 525-A was a competitive inhibitor of aminopyrine demethylation in rat and mouse microsomes; noncompetitive inhibition was found in the rabbit. Similar results were obtained with DPEA, chlorcyclizine, and U-16392-A, although the latter two compounds sometimes produced a mixed-type inhibition. In the case of hexobarbital metabolism, competitive inhibition by all four compounds was seen in rat liver microsomes whereas rabbit liver microsomes showed noncompetitive inhibition. Quantitative differences were also observed.

The method of studying the action of inhibitors may play a role in kinetic experiments. Cho, Hodshon & Brodie (78) have studied the kinetics of the inhibition of ethylmorphine demethylation by phenylbutazone when the inhibitor was either added directly to microsomal fractions or administered to animals prior to isolation of microsomes. In the former case, competitive inhibition was observed while in the latter case noncompetitive inhibition was obtained. Gel filtration experiments suggested that the noncompetitive inhibition was attributable to an irreversible binding of phenylbutazone or a metabolite to microsomes.

The effect of varying the time interval between the administration of the inhibitor and the drug being inhibited has been studied. Stitzel, Anders & Mannering (74) observed that SKF 525-A, SKF 8742-A, and SKF 26754-A were approximately equipotent inhibitors of hexobarbital metabolism in vivo when administered 45 min prior to hexobarbital. However, when the

time interval was increased to 5 hr, SKF 525-A proved to be a more potent inhibitor of hexobarbital metabolism than SKF 8742-A, which was more effective than SKF 26754-A. The finding that the three inhibitors were nearly equipotent when the time interval was 45 min was not unexpected in view of in vitro inhibition and metabolism studies (40, 41). These experiments had demonstrated that the K_i of SKF 525-A, SKF 8742-A, and SKF 26754-A were 6.0, 3.6, and 1.6 $\times$ 10^{-6} M, respectively. If these values were used to predict inhibitory capacity in vivo, the three compounds would be expected to show about equal potency. The differences observed between the three inhibitors when the time interval was 5 hr were explained on the basis of metabolism experiments (41). SKF 8742-A has been shown to be a metabolite of SKF 525-A; in addition SKF 8742-A undergoes N-deethylation to yield SKF 26754-A (41). Thus, metabolism of SKF 525-A would not result in the termination of its inhibitory activity since the metabolite (SKF 8742-A) is also an inhibitor. By the same token, SKF 8742-A also yields an inhibitory metabolite (SKF 26754-A). SKF 26754-A may be metabolized to a compound lacking appreciable inhibitory properties or may be readily excreted. Thus, SKF 525-A undergoes two N-dealkylations before becoming SKF 26754-A and was the most potent inhibitor at the 5 hr time interval. SKF 8742-A, which undergoes only one N-dealkylation to become SKF 26754-A, was intermediate in potency and SKF 26754-A was least potent.

A number of investigators have noted that, following an initial inhibitory phase, many inhibitors produce a stimulation of drug metabolism. Rümke & Bout (79) observed that several drugs prolong hexobarbital narcosis when given shortly before the administration of hexobarbital; these same drugs shorten the duration of hexobarbital action when given one or more days prior to the administration of hexobarbital. Hydroxyzine, chlorpromazine, promazine, orphenadine, phenobarbital, glutethimide, urethane, trimethadione, methoin, primidone, iproniazid, SKF 525-A, and carbutamide shortened the duration of hexobarbital narcosis when given 24–96 hr before; reserpine, azacyclonol, tolbutamide, and chlorpropamide produced no decrease in hexobarbital narcosis.

Serrone & Fujimoto (80) showed that N-methyl-3-piperidyl-(N,N′)-diphenylcarbamate (MPDC) given 1–12 hr before hexobarbital prolonged sleeping time by inhibiting the metabolism of the barbiturate; when given 24–48 hr before hexobarbital, the sleeping time was shortened due to an increased rate of hexobarbital metabolism. The same authors observed similar effects with N-ethyl-3-piperidylbenzilate (EPB, JB 318), SKF 525-A, iproniazid, and nikethamide (81). Kato, Chiesara & Vassanelli (68, 82) also showed that known inhibitors of drug metabolism stimulated the rate of metabolism of meprobamate, strychnine, and hexobarbital. Buller et al (83) observed that 4,5-dihydro-6-methyl-2[2-(4-pyridyl)-ethyl]-3-pyridazone (U-320) both inhibited and stimulated hexobarbital hypnosis; Neubert & Herken (27) obtained similar results with diethylaminoethyl esters of phenyldiallylacetic acid. Neumann, Miya & Bousquet (84) observed that a tol-

erance developed to the effects of SKF 525-A, thus confirming the earlier observation of Cook, Toner & Fellows (20).

Anders & Mannering (85) studied the stimulatory effect of SKF 525-A and Lilly 18947 on microsomal drug metabolism. It was found that SKF 525-A and Lilly 18947 induced microsomal enzyme activity as measured by the N-dealkylation of these compounds themselves as well as that of ethylmorphine. Furthermore, SKF 525-A proved to be an effective inhibitor of hexobarbital metabolism in SKF 525-A and phenobarbital treated rats. This was attributed to the formation of inhibitory metabolites of SKF 525-A.

Inhibition of drug metabolism in man.—In view of the common practice of multiple drug administration to humans, it is not surprising that instances of drug inhibition have been reported. Bishydroxycoumarin has been reported to inhibit the metabolism of diphenylhydantoin (86), tolbutamide (87), and chlorpropamide (88). In the case of diphenylhydantoin, the inhibition of metabolism led to toxic effects. Carter (89) and Solomon & Schrogie (90) demonstrated that phenyramidol inhibited the metabolism of anticoagulants in human subjects. Inhibition of diphenylhydantoin metabolism by disulfiram (91–93), isoniazid (94, 95), and phenyramidol (96) has been observed. Tolbutamide metabolism in man was observed to be inhibited by sulphaphenazole and phenylbutazone (97) as well as chloramphenicol (98). The latter compound also inhibited the metabolism of diphenylhydantoin and dicoumarol (98).

Special effects of SKF 525-A.—A review of the inhibition of microsomal drug metabolism could scarcely be considered complete without noting some of the properties of SKF-525-A in addition to its well known ability to inhibit microsomal oxidations.

In addition to inhibiting microsomal drug oxidations, SKF 525-A has been shown to inhibit glucuronyl transferase (24, 25, 99, 100) and monoamine oxidase (101, 102). No inhibition of acetylcholinesterase was observed (101). Inhibition of cholesterol biosynthesis by SKF 525-A has been reported by several workers (103–108).

Dick et al (106) and Gaitondé & Borison (109) have noted toxic effects of SKF 525-A. The former workers (106) observed that SKF 525-A produced a marked fatty infiltration of the liver, which was rapidly reversible upon withdrawal of the compound, while the latter (109) reported respiratory and gastrointestinal disturbances. Effects seen after administration of SKF 525-A were attributed to a combination of central and peripheral effects.

SKF 525-A has been shown to have diverse pharmacological effects. Included are inhibition of muscle contractions (110) and potentiation of neuromuscular blocking drugs (111–116). In addition, SKF 525-A was reported to act peripherally as either a parasympatholytic or a sympathomimetic agent or both (101).

Magus & Fouts (117) observed that SKF 525-A produced a stimulatory action in vivo on hepatic tryptophan pyrrolase activity. Evidence was presented indicating that some form of pituitary activation was involved, possibly related to the action of antidiuretic hormone (118). Kupfer & Peets (119) reported that SKF 525-A potentiated the cortisol induction of hepatic tyrosine transaminase.

SKF 525-A has been shown to produce a concentration dependent hemolysis or stabilization of the red cell membrane (34). Florence (35) observed that SKF 525-A is more surface-active than chlorpromazine.

Rogers, Dixon & Fouts (120) noted that SKF 525-A lowered hepatic glycogen levels. High concentrations of SKF 525-A inhibited glycogen synthetase activity but failed to alter glycogen breakdown. These studies were later extended by Wooles & McPhillips (121) who showed that, while SKF 525-A did lower hepatic glycogen levels, this effect was not related to the inhibition of drug metabolism due to SKF 525-A.

Finally, SKF 525-A has been shown to inhibit autoregulation of renal blood flow and to diminish responsiveness of the kidney to norepinephrine (122). Other studies (123) showed that SKF 525-A produced a natruretic effect when infused into the renal artery. The natruretic effect of SKF 525-A was additive when other known natruretic agents were co-administered (124). The intraperitoneal administration of SKF 525-A has been reported to produce an antidiuretic effect (125).

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