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HEPATIC GLUTATHIONE AND HEPATOTOXICITY

EFFECTS OF CYTOCHROME P-450 COMPLEXING COMPOUNDS SKF 525-A, L-α ACETYLMETHADOL (LAAM), norLAAM, AND PIPERONYL BUTOXIDE*

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Abstract—Four compounds forming metabolic intermediate complexes with cytochrome P-450 *in vitro* were studied for their effects on hepatic glutathione in the mouse. All four compounds depleted glutathione within 1–3 hr after administration. The effect was transient for piperonyl butoxide while lasting at least 24 hr for other compounds. Induction of the mixed-function oxidase system by phenobarbital had no effect on the glutathione-depleting actions of the compounds, but induction with 3-methylcholanthrene abolished the depletion seen with piperonyl butoxide and SKF 525-A. For SKF 525-A, L-α-acetylmethadol (LAAM) and norLAAM, the persistent lowering of glutathione was paralleled by elevations in serum glutamic-pyruvic transaminase (SGPT) activity. This depletion of glutathione and subsequent elevations in SGPT were found to be strain and species dependent for SKF 525-A, LAAM and norLAAM. Compounds which complex with cytochrome P-450 *in vitro* may increase drug toxicities *in vivo* by mechanisms other than inhibition of oxidative drug metabolism.

Glutathione plays an important role in normal hepatocellular function. These important cellular functions include: the maintenance of sulfhydryl groups of proteins; the destruction of hydrogen peroxide, lipid peroxides, and free radicals; translocation of amino acids across cell membranes; the detoxification of foreign compounds; and the biotransformation of drugs [1]. Thus, perturbations of hepatocellular glutathione levels can alter normal hepatocellular integrity. The importance of glutathione in maintaining normal cellular integrity in isolated hepatocytes has been reported previously [2]. Therefore, compounds which deplete glutathione not only compromise cellular defenses against attack by reactive molecules but they may also have profound effects on normal hepatocellular functions.

In recent years, it has been shown that during the microsomal metabolism of some compounds a reactive metabolic intermediate (MI) is formed that binds or complexes to cytochrome P-450 and inhibits further drug metabolism [3–6]. This MI complex formation was the first proposed biochemical mechanism to explain how SKF 525-A was capable first of competitive and then later of noncompetitive inhibition of drug metabolism as had been described by Rogers and Fouts [7] a decade earlier. The *in vivo* occurrence of these inhibitory MI complex forming reactions was later suggested when SKF 525-A produced inhibitory MI complexes to half of the cytochrome P-450 present in the rat [8], and amphetamine compounds were reported to have formed MI complexes in isolated hepatocytes [9, 10]. During the later studies, it was observed that the norbenzphetamine MI complex formation coincided with a depletion of intracellular glutathione and that glutathione prevented MI complex formation to microsomal cytochrome P-450. Thus, it was concluded that glutathione may be important in the regulation or manifestation of this phenomenon [9].

The implication that a compound forming MI complexes *in vivo* may also deplete glutathione is of great physiologic and practical pharmacologic importance. As stated above, glutathione is central to many cellular functions of which the most immediately important is the detoxification of reactive or toxic intermediates formed during cellular respiration or biotransformation of xenobiotics. Thus, prolonged or significant perturbation of the glutathione pool may lead directly to hepatotoxicity or may potentiate the hepatocellular damage produced by other compounds. Since some of the MI complex forming compounds are of prevalent clinical use (e.g. propoxyphene, imipramine, and diphenhydramine, see Ref. 6) or of widespread use in the pharmacologic evaluation of the metabolism of other compounds (SKF 525-A, Lilly 18947, and piperonyl butoxide), it was of great interest to determine whether or not glutathione depletion might be a general phenomenon of this class of inhibitory compounds *in vivo*. Such a finding would imply that these compounds are potentially hepatotoxic, especially in multiple drug therapy with other hepatotoxins such as acetaminophen, and would suggest that alternative mechanisms other than decreased drug metabolism

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may be involved when explaining the toxic drug–drug interactions of these compounds. Therefore, the purpose of this study was to examine several MI complex forming drugs outside of the amphetamine family to determine whether or not they also caused a depletion of glutathione and to determine the ramifications, if any, of such an effect.

MATERIALS AND METHODS

Chemicals. SKF 525-A was provided by Smith, Kline & French Laboratories (Philadelphia, PA). The L-alpha-acetylmethadol (LAAM) and norLAAM were gifts of the National Institute on Drug Abuse. Piperonyl butoxide was donated by the Fairfield American Corp. (Medina, NY). Serum glutamic-pyruvic transaminase (SGPT) kits and 3-methylcholanthrene were obtained from the Sigma Chemical Co. (St. Louis, MO), and sodium phenobarbital was purchased from the Mallinkrodt Chemical Works (St. Louis, MO).

Animal pretreatments. Male animals were obtained from Harlan Industries (Indianapolis, IN) and were housed for 1 week before use. The species used in this study included Swiss origin mice (22–26 g), Golden Syrian hamsters (70–80 g), and Fischer 344 and Sprague–Dawley rats (200–250 g). The animals were fed *ad lib*. and exposed to a constant 12-hr light/dark cycle. The piperonyl butoxide and 3-methylcholanthrene were dissolved in corn oil while all other compounds were administered in saline. LAAM and norLAAM were given orally in a total volume of 0.25 ml or less; all other compounds were injected i.p. in similar volumes. The phenobarbital induction consisted of four daily doses of 70 mg/kg, the 3-methylcholanthrene was given for 3 days at 40 mg/kg, and all animals were used 24 hr after the last dose.

Assays. Reduced glutathione or total soluble SH content per gram of tissue was assayed by a modification of the spectrophotometric technique reported by Sedlack and Lindsay [11], and all time intervals were measured against the appropriate control because glutathione levels in the liver are known to fluctuate over a 24-hr period [12]. This method is based on the reductive cleavage of 5,5′-dithiobis-2-nitrobenzoic acid by SH groups to yield a yellow color with an absorbance maximum at 412 nm. Aliquots of the TCA–sodium EDTA homogenate were centrifuged at 1000 *g* for 15 min, and 0.4 ml of the protein-free supernatant fraction was mixed with 4.55 ml of a sodium phosphate buffer (0.01 M, pH 8.0). To this, 0.05 ml of 0.01 M 5,5′-dithiobis-2-nitrobenzoic acid in a sodium phosphate buffer (0.01 M, pH 7.0) was added, and absorbance of the resultant yellow color was detected at A_{412} within 2 min by using a Hitachi 100-20 spectrophotometer. Results were calculated as micrograms of glutathione (G-SH) per gram of wet weight of tissue and converted to percentage changes after calibration with standards prepared from pure G-SH as the SH donor. The serum glutamic-pyruvic transaminase was measured by the method of Reitman and Frankel [13]. The results of all assays are reported as the means and standard deviation. Differences between means were determined using Student's *t*-test.

RESULTS

SKF 525-A, LAAM, norLAAM and piperonyl butoxide significantly depleted hepatic glutathione, and the depletion was dose and time dependent. For example, SKF 525-A, 25 mg/kg, caused maximal glutathione depletion at around 1 hr and glutathione levels returned to normal by 2 hr (Fig. 1). On the other hand, at 2 hr a 50 mg/kg dose maintained a constant depletion of glutathione during this same time period, whereas with a 100 mg/kg dose the glutathione concentrations were still decreasing at 3 hr. SKF 525-A, LAAM and norLAAM at doses above 25 mg/kg, and at 100 mg/kg or higher for piperonyl butoxide, induced a significant depletion of hepatic glutathione. Doses that induced nearly maximum depletion of glutathione were 100 mg/kg of SKF 525-A, 75 mg/kg of LAAM, 60 mg/kg of norLAAM, and 400 mg/kg of piperonyl butoxide. The effect of piperonyl butoxide on hepatic glutathione was temporally different from the other compounds tested. Maximum glutathione depletion, induced by piperonyl butoxide, occurred between 1 and 3 hr and the glutathione levels returned to normal within 5 hr (Fig. 2). The other compounds, however, appeared to reach a level of constant depletion within the first 3 hr and, quite surprisingly,

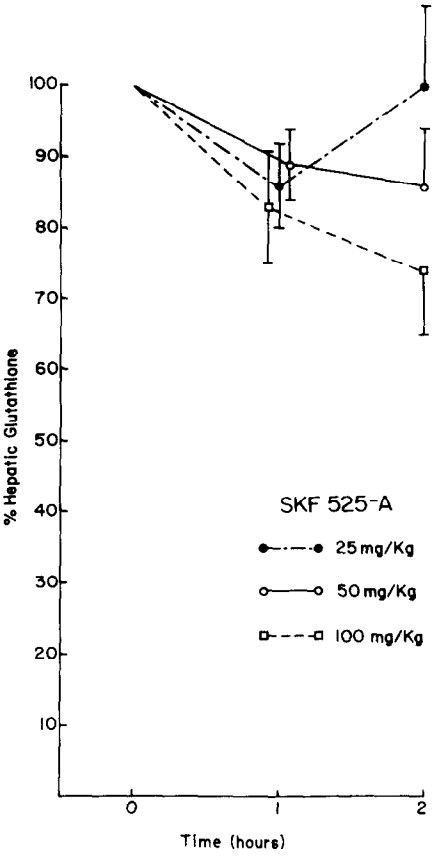

Fig. 1. Dose-dependent depletion of hepatic glutathione in the mouse. SKF 525-A was injected intraperitoneally at various doses. Six mice were killed for each dose and time interval. Each point is the mean calculated as the percent of the control mean bracketed by the S.D.

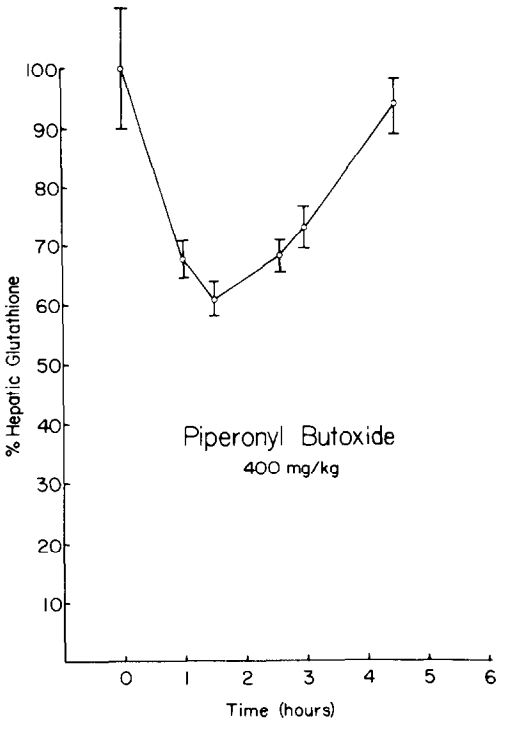

Fig. 2. Time course of hepatic glutathione depletion by piperonyl butoxide. Piperonyl butoxide was injected intraperitoneally at a dose of 400 mg/kg. Each point is the mean and S.D. of six to twelve mice. The values have been converted to percent of the control mean to ease comparisons.

recovery was not evident within the first 6 hr. For example, the effect of SKF 525-A on hepatic glutathione was maximum within 1 hr following treatment, but the depletion remained for 6 hr following treatment (Fig. 3). Unlike SKF 525-A, LAAM-induced depletion of hepatic glutathione was not as rapid (Fig. 3) and a maximum decrease did not occur until about 4.5 hr after administration. LAAM had lowered hepatic glutathione by about 45% from 4.5 to 6 hr following treatment. However, norLAAM, a major metabolite of LAAM, also significantly depleted hepatic glutathione (Fig. 3), but the norLAAM-induced depletion began more rapidly while the extent and time of depletion seen was similar to that of LAAM.

SKF 525-A, LAAM, and norLAAM all significantly lowered hepatic glutathione within the first 3 hr following administration and continued to depress glutathione levels for more than 24 hr following a single administration of each compound. Although the depletion of glutathione was never much greater than 50%, the duration of this glutathione depletion was surprisingly long and the drug-induced glutathione loss was large when considered in the context of a normal 24-hr cycle.

This drug-induced depletion of hepatic glutathione was evaluated in other species. SKF 525-A, LAAM- and norLAAM-induced depletion of hepatic glutathione occurred in several species but this effect was both species and strain dependent (Table 1). These drugs significantly lowered hepatic glutathione in Golden Syrian hamsters and Fischer 344 rats, but they produced no significant changes in Sprague–Dawley rats or guinea pigs.

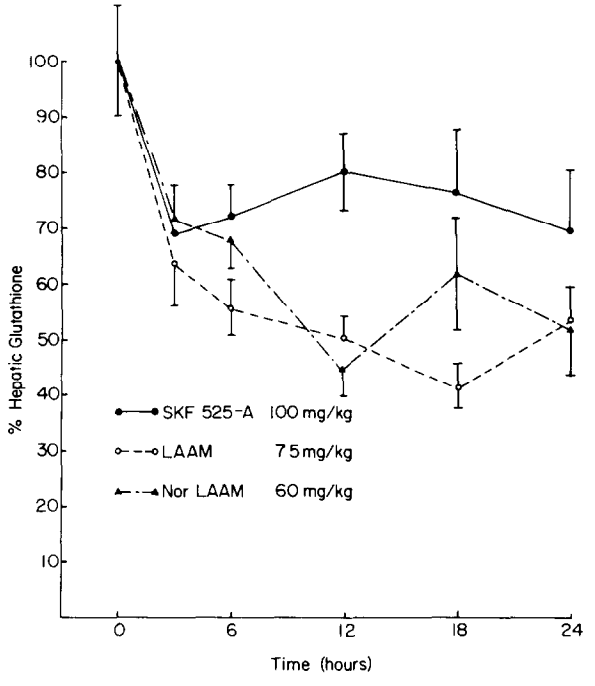

Fig. 3. Effects of SKF 525-A, LAAM and norLAAM on hepatic glutathione for 24 hr. Hepatic glutathione in the mouse was determined at various time intervals after the administration of SKF 525-A, LAAM and norLAAM. Each point is the mean and S.D. of twelve mice. The values have been converted to percent of the control mean.

Table 1. Effects of SKF 525-A, LAAM and norLAAM on hepatic glutathione in various species*

	Glutathione (mg/g liver)			
	Golden Syrian hamsters	Fischer 344 rats	Sprague–Dawley rats	Guinea pigs
Untreated	2.52 ± 0.25	2.02 ± 0.16	1.99 ± 0.25	2.86 ± 0.30
SKF 525-A, 100 mg/kg	1.99 ± 0.16†	1.63 ± 0.13†	1.94 ± 0.09	2.47 ± 0.22
LAAM, 50 mg/kg	1.97 ± 0.12†	0.94 ± 0.23†	2.00 ± 0.39	
norLAAM, 45 mg/kg		1.01 ± 0.32†	2.48 ± 0.29	2.49 ± 0.20

* Six to twelve animals were killed approximately 3 hr after the administration of each compound. The values are means ± S.D.

† $P < 0.05$, significantly different from corresponding control.

These species–strain differences are consistent with a mixed-function oxidase-dependent bioactivation. Phenobarbital, an inducing agent which increases MI complex formation *in vitro*, caused no significant change in any of the drug-induced depletions of hepatic glutathione (Fig. 4). However, 3-methylcholanthrene induction, which abolishes MI complex formation *in vitro* for the alkylamine compounds, eliminated the glutathione depletion seen with both piperonyl butoxide and SKF 525-A. Thus, it appears that oxidative metabolism and, possibly, MI complex formation may play roles in selected drug-induced hepatic glutathione depletion.

SKF 525-A, LAAM and norLAAM which caused a prolonged lowering of hepatic glutathione also elevated SGPT activity parallel to the glutathione depletion (Table 2). On the other hand, piperonyl butoxide which did not cause a prolonged depletion of glutathione likewise did not significantly elevate SGPT levels. Piperonyl butoxide had no effect on SGPT activity even if glutathione was completely depleted prior to administration by diethylmaleate (data not shown). Similarly, drug-induced hepatocellular injury was observed only in those species and strains in which hepatic glutathione depletion was observed (Table 3). SKF 525-A, LAAM and norLAAM significantly depleted hepatic glutathione in hamsters, mice, and Fischer 344 rats and also

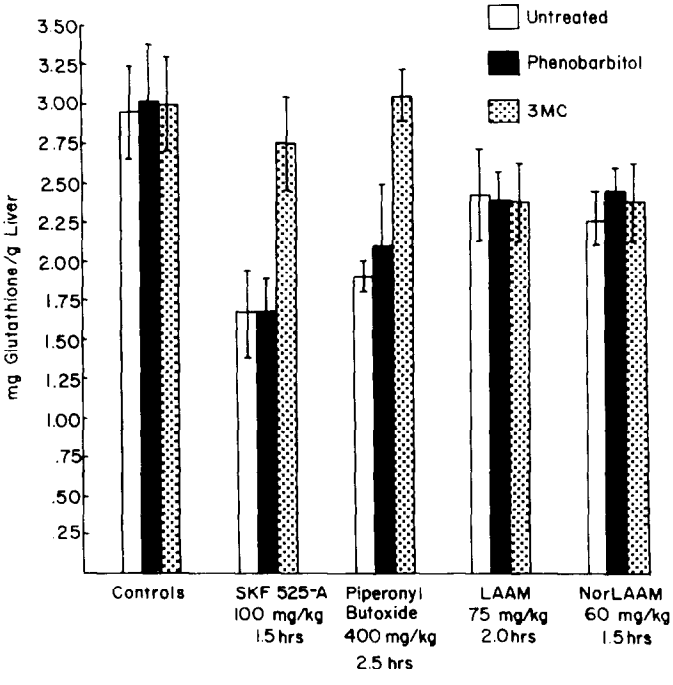

Fig. 4. The effect of mixed-function oxidase induction on the hepatic glutathione depletion of SKF 525-A, LAAM, norLAAM, and piperonyl butoxide. Control values are the mean and S.D. of thirty mice. All other values are the mean and S.D. of six to eight mice.

Table 2. Effects of SKF 525-A, LAAM, norLAAM and piperonyl butoxide on mouse SGPT levels*

Hours after administration	SGPT (units/ml) SKF 525-A (100 mg/kg)	LAAM (75 mg/kg)	norLAAM (60 mg/kg)	Piperonyl butoxide (400 mg/kg)
Untreated	34 ± 12	34 ± 12	34 ± 12	34 ± 12
3	272 ± 86†			39 ± 7
6	237 ± 108†	146 ± 68†	659 ± 338†	39 ± 6
18	381 ± 153†	174 ± 54†	141 ± 37†	
24	179 ± 34†	182 ± 101†	239 ± 93†	

* Six to twelve animals were killed at each time interval. All values are means ± S.D. SKF 525-A and piperonyl butoxide were injected intraperitoneally, while LAAM and norLAAM were administered orally.
† $P < 0.05$, significantly different from corresponding control.

Table 3. Effects of SKF 525-A, LAAM and norLAAM on SGPT levels in various species*

	SGPT (units/ml) Golden Syrian hamsters	Fischer 344 rats	Sprague–Dawley rats	Guinea pigs
Untreated	28 ± 11	48 ± 12	18 ± 5	25 ± 5
SKF 525-A, 100 mg/kg	181 ± 96†	172 ± 84†	43 ± 9	38 ± 6
LAAM, 50 mg/kg		1000 ± 327†	23 ± 3	27 ± 11
norLAAM, 45 mg/kg		777 ± 437†	26 ± 4	41 ± 25

* Six to ten animals were killed approximately 3 hr after the administration of each compound.
† $P < 0.05$, significantly different from corresponding control.

significantly elevated SGPT levels in these animals. SKF 525-A, LAAM and norLAAM did not significantly deplete hepatic gluthione in Sprague–Dawley rats and guinea pigs and likewise did not significantly elevate SGPT levels in these animals. Thus, a relationship appeared to exist between the drug-induced depletion of hepatic glutathione and the drug-induced hepatocellular injury.

DISCUSSION

The MI complex forming compounds piperonyl butoxide, SKF 525-A, LAAM and norLAAM all lowered hepatic glutathione. The persistent lowering of glutathione was unexpected. Other compounds which are known to interact with glutathione, for example acetaminophen [14] or bromobenzene [15], or the sulfoxime compounds which inhibit glutathione synthesis [16], only lower glutathione for a few hours. The glutathione-lowering properties of SKF 525-A are of particular interest because of the extensive use of this compound as a tool to investigate the role of biotransformation in chemical-induced toxicities. SKF 525-A was both a competitive inhibitor of mixed-function oxygenases as well as a depletor of hepatic glutathione. This confounding factor could explain the enhancement of certain chemical-induced hepatotoxicities by SKF 525-A that may not be biotransformation dependent. For example, SKF 525-A enhanced the hepatotoxicity of acetaminophen following pretreatment 12 hr prior to the drug administration. This effect was not seen if drug treatment was 3 hr prior to drug administration. There is a correlation between SKF 525-A depletion of glutathione and enhancement of this drug-induced hepatotoxicity.

The MI complexing compounds represent two different chemical classes. Piperonyl butoxide is of the methylenedioxphenyl group of compounds, while SKF 525-A, LAAM and norLAAM are of the alkylamine group. The functional groups reacting

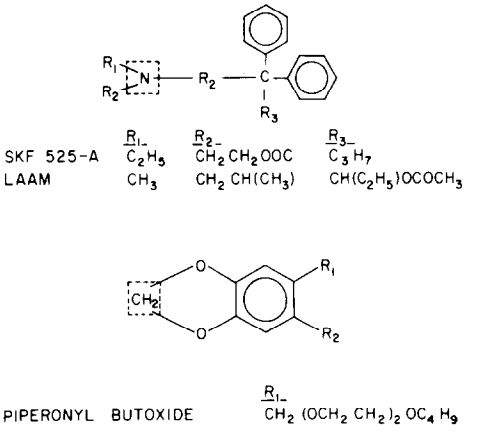

Fig. 5. Chemical structures of SKF 525-A, LAAM and piperonyl butoxide.

with cytochrome P-450 [17, 18] are noted by the broken lines in Fig. 5. The chemical structures of the MI complexing compounds differ as well as their routes of biotransformation. However, the nature of the proposed reactive metabolic intermediates which form MI complexes may be the common link leading to the glutathione depletion. Such a conclusion would be consistent with other reports. Hirata *et al.* [9] found that glutathione prevents microsomal MI complex formation with amphetamine compounds and concluded that glutathione was reacting with the MI complex forming metabolic intermediate. MI complex formation is dependent on mixed-function oxygenase activity. Franklin [3, 6] has demonstrated repeatedly that 3-methylcholanthrene induction lowers the rate of MI complex formation *in vitro* for the alkylamine class of MI complex forming compounds, which would explain why 3-methylcholanthrene induction eliminated the glutathione depletion induced by the alkylamine SKF 525-A. However, it does not explain why 3-methylcholanthrene blocked the piperonyl butoxide-induced depletion of glutathione. The dependency of SKF 525-A MI complex formation on mixed-function oxygenase activity may be dependent on formation of an *N*-hydroxy metabolite [10]. *N*-Oxidation yields a metabolite which can either react with glutathione or hydrolyze to an *N*-hydroxy metabolite which is then capable of further metabolism to the MI complexing intermediate. If nitrogen oxidation yields a hydroxylamine directly, then only MI complex formation is detected. Thus, there may not be a direct relationship between MI complex formation and glutathione depletion. The relationship will depend on the dominant biotransformation pathway. The fact that 3-methylcholanthrene blocked piperonyl butoxide-induced depletion of glutathione but did not affect MI complex formation further supports this explanation. Other explanations for the drug-induced lowering of glutathione are an accelerated oxidation of glutathione or an enhanced degradation or inhibition of synthesis. Although these are possibilities, the evidence currently supports a direct chemical interaction as the mechanism for the drug-induced lowering of glutathione.

SKF 525-A, LAAM and norLAAM elevated SGPT levels for the approximate time period of observed glutathione depletion. This drug-induced hepatocellular injury may have been produced by the chemically reactive cytochrome P-450 complexing intermediates, or by a closely related metabolite [10], which is sufficiently electrophilic to interact with glutathione [10] and therefore also capable of inducing hepatocellular injury. Glutathione depletion may have been seen because it was functioning in its role as a scavenger of reactive toxic metabolic intermediates. Alternatively, the persistent glutathione depletion produced by these compounds may have diminished the usual protective role of glutathione in detoxifying endogenous peroxides, and other chemically reactive substances, and thereby disturbed its function in maintaining membrane integrity. Another possibility is that SKF 525-A, LAAM and norLAAM were bioactivated to toxic metabolites and directly damaged hepatocytes, causing a loss of SGPT and a depletion of glutathione. Regardless of the mechanism causing the hepatocellular damage, however, the MI complex forming compounds were inherently hepatotoxic. Again, caution must be exercised when using SKF 525-A as an investigative tool to determine whether the parent compound or a metabolite is responsible for a particular chemically induced hepatotoxicity, since SKF 525-A is itself hepatotoxic and further lowers hepatic glutathione levels. These effects may enhance the toxicity of a compound, as was found to be the case for acetaminophen, but by a mechanism other than alteration of mixed-function oxygenases. Therefore, the precise interpretation of the effect of SKF 525-A pretreatment when altering the toxicity of a particular drug requires knowledge of its effect on metabolism as well as its direct effects on the target organ.

The four MI complex forming compounds that were studied depleted hepatic glutathione *in vivo*, and the persistent depletion produced by the alkylamine compounds led to hepatic injury. These effects occurred in several species and are, therefore, an important consideration in all animal models in which these compounds are used. MI complex forming compounds may act, besides inactivating cytochrome P-450, to deplete glutathione and elicit membrane damage. Specifically, SKF 525-A alters normal hepatocellular physiology in addition to inhibiting specific cytochrome P-450-dependent oxidative metabolism. Therefore, the use of SKF 525-A as a tool to study the relationship between the alteration of oxidative metabolism and the toxicity of a drug may be inappropriate or lead to ambiguous results. The conclusions from this type of study must now at least be tempered, for one cannot assume that these results necessarily implicate the parent molecule as the toxic form of the compound. It must also be mentioned that SKF 525-A, LAAM, and norLAAM are all narcotic agonists [19, 20] as well as MI complex forming drugs. So, while this study intended to examine a possibly general phenomenon of MI complex forming drugs, i.e. glutathione depletion, it may have serendipitously discovered a hepatotoxic potential that may well be an interaction common to many narcotics. Morphine has already been reported to elevate SGPT activity [21], and studies in this laboratory indicate that it does lower hepatic glutathione. The need for a better understanding of the glutathione-depleting actions and inherent hepatotoxicity of these compounds is clear. Specifically, it is essential to determine whether these actions are related to the narcotic family of compounds, the MI complexing family of compounds, or are of consequence to only these drugs.

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