Biochemical Pharmacology, Vol. 29, pp. 3239–3244.

0006-2952/80/1215-3239 $02.00/0

BLEOMYCIN TOXICITY

ALTERATIONS IN OXIDATIVE METABOLISM IN LUNG AND LIVER MICROSOMAL FRACTIONS*

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(*Received* 13 *November* 1979; *accepted* 16 *June* 1980)

Abstract—Separate groups of male rats received low doses (5 units) or high doses (15 units) of bleomycin i.p. twice weekly for 1.5, 3 or 6 weeks. The susceptibility of tissue lipid to peroxidation and the activities of mixed function oxidations were examined in microsomal fractions prepared from lung and liver. ADP–Fe (III)-initiated lipid peroxidation was stimulated in lung microsomal fractions only in animals treated with high-dose bleomycin for 1.5 weeks, whereas a 2- to 4-fold enhancement was observed in liver preparations from all bleomycin-treated animals. Microsomal ADP–Fe (III)-initiated lipid peroxidation was inhibited, however, by the *in vitro* addition of bleomycin in both lung and liver preparations, but this inhibition was an artifact resulting from the chelation of Fe (III) by bleomycin. Soybean lipoxygenase I-initiated microsomal lipid peroxidation, which does not require added iron, was unaffected by bleomycin in lung preparations but was inhibited in liver. Following *in vivo* treatment, lung microsomal hydrogen peroxide generation was inhibited by 1.5 weeks of high-dose bleomycin treatments, whereas benzphetamine *N*-demethylation was unchanged. These cytochrome P-450-dependent reactions were both suppressed, however, in liver microsomal fractions. *In vitro*, both reactions were also inhibited by bleomycin in liver but not in lung microsomal fractions. The lack of effect of *in vitro* bleomycin treatments on superoxide generation in lung or liver preparations suggests that the NADPH cytochrome P-450 reductase component of the mixed function oxidase system was not affected. Minimal alterations in lipid peroxidizability and mixed function oxidase activities in lung microsomal fractions of bleomycin-treated animals suggest that the insensitivity could be due to: (1) the site of toxicity not being at the level of the endoplasmic reticulum; or (2) the target of bleomycin toxicity being limited to a small population of pulmonary cell types. Even though the liver is not susceptible to bleomycin toxicity, the hepatic microsomal mixed function oxidase system is highly sensitive to this chemical.

Bleomycin is a glycopeptide antibiotic that is isolated as a copper (II)-chelate from the culture broth of *Streptomyces verticillus* [1]. The copper-free preparation of bleomycin is used in combination with other antineoplastic agents and radiotherapy in cancer chemotherapy [2, 3]. Unfortunately, the frequent occurrence of severe pulmonary toxicity has restricted the clinical use of this drug [4, 5].

Several studies have suggested that bleomycin exerts its antitumor activity by binding to DNA [6–8] and causing strand scission [9, 10]. This disruptive process is stimulated in the presence of reducing or oxidizing agents [11], free-radical generating systems [12] or catalytic amounts of Fe (II) and oxygen [13, 14]. It remains unclear how the Fe (II)–bleomycin complex is involved in DNA degradation, although it has been proposed that oxidation of the Fe (II)–bleomycin complex generates reactive oxygen species such as superoxide and hydroxyl radicals [15–17].

Activated oxygen species are known to initiate chain reactions which result in peroxidation of membrane lipids [18, 19], leading to loss of membrane structure and membrane-bound enzyme activities [20, 21]. Inactivation of microsomal drug-metabolizing enzyme systems and destruction of cytochrome P-450 have been observed concurrently with lipid peroxidation [22, 23]. Moreover, alteration in pulmonary microsomal mixed function oxidase activities following acute doses of oxidants may indicate toxicity [24].

We have previously established an experimental model of pulmonary toxicity in rats by repeated administration of bleomycin (5 units, twice weekly) for up to 6 weeks [25, 26]. This communication reports *in vivo* and *in vitro* effects of bleomycin on lipid peroxidizability, benzphetamine *N*-demethylation, and hydrogen peroxide generation in lung microsomal fractions. Liver microsomal fractions

* This work was supported, in part, by NIH Grant ESHL 01365. Portions were presented at the joint meeting of the American Society of Pharmacology and Experimental Therapeutics and the Society of Toxicology, August 13–17, 1978, in Houston, TX, and at the 64th annual meeting of the Federation of American Societies for Experimental Biology, April 13–18, 1980, in Anaheim, CA.

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were also examined to establish if observed changes were specific to the lung or were generalized effects.

MATERIALS AND METHODS

Chemicals. Bleomycin (mol. wt ~1400) was supplied by Bristol Laboratories, Syracuse, NY, as the copper-free sulfate salt, 15 biological units per ampule (1.2 to 1.7 units/mg weight), containing 55–70% bleomycin A_2 and 25–32% bleomycin B_2. Benzphetamine hydrochloride was provided by The Upjohn Co., Kalamazoo, MI (Lot No. 352-FS). Soybean lipoxygenase I, Torula yeast glucose-6-phosphate dehydrogenase and bovine liver catalase were purchased from the Sigma Chemical Co., St. Louis, MO. 2-Thiobarbituric acid (TBA) was from Eastman Organic Chemicals, Rochester, NY. Other chemicals were reagent grade.

Treatment of animals. Male, CD strain Sprague–Dawley rats (180–200 g) were obtained from the Charles River Breeding Laboratories, Wilmington, MA. Low doses (5 units) or high doses (15 units) of bleomycin (in 0.5 ml of 0.9% sodium chloride) were administered i.p. twice weekly to separate groups for 1.5, 3 or 6 weeks. Controls received an equal volume of 0.9% saline. The mortality was 10 per cent for the 6-week low-dose group [26]. Due to the high mortality (>80 per cent) after 6 weeks of the high dose, this treatment group was not available for investigation.

Preparation of microsomal fractions. Twenty hours after the final injection, animals were killed by cervical fracture and exsanguinated. The lung or liver was removed, minced, and washed three times with ice-cold 0.15 M KCl containing 0.02 M Tris buffer, pH 7.4 (Tris–KCl buffer). The washed mince was homogenized for 15 sec twice in 3 vol. of ice-cold Tris–KCl buffer with a Super Dispax Tissumizer model SDT-182N (Tekmar, Cincinnati, OH) at 12,000 rpm. The resulting homogenate was centrifuged at 9,000 *g* for 15 min at 5°. The supernatant fraction was centrifuged at 165,000 *g* for 45 min at 5°. The pellet was suspended in Tris–KCl buffer and resedimented at 165,000 *g* for an additional 45 min at 5°. The protein concentration of the microsomal pellet was measured by the method of Sutherland *et al.* [27] with bovine serum albumin as standard.

Estimation of lipid peroxidizability. Fe (III)–ADP dependent peroxidation was initiated by adding 0.1 ml of microsomal protein (10 mg/ml) to 0.9 ml of a reaction mixture containing 0.5 mM NADPH, 4 mM ADP, and 12 μM $FeCl_3$ in Tris–KCl buffer, pH 7.4, and incubating it at 37° for 20 min; one ml of 10% (w/v) trichloroacetic acid was added to terminate the reaction. The lipid peroxidation products were measured by the thiobarbituric acid reaction as described by Ernster and Nordenbrand [28]. For soybean lipoxygenase I-initiated lipid peroxidation, 0.1 ml of microsomal protein (10 mg/ml) was incubated with 0.9 ml of Tris–KCl buffer containing 0.1 mg soybean lipoxygenase I (EC 1.13.11.12) (sp. act. 57.6 μmoles of linoleic acid peroxidized per mg of enzyme per min at 20°, pH 9.0) at 37° for 20 min.

Determination of benzphetamine N-demethylation. Benzphetamine hydrochloride (2 mM) was incubated at 37° for 20 min with an NADPH-generating system and 1 mg of microsomal protein in 1 ml of Tris–KCl buffer, pH 7.4. Microsomal benzphetamine *N*-demethylase activity was determined by the colorimetric assay of formaldehyde production with Nash's reagent [29].

Estimation of hydrogen peroxide. The microsomal generation of hydrogen peroxide was determined by the conversion of methanol to formaldehyde [30].

Estimation of superoxide anion. The microsomal generation of the superoxide anion radical was followed by the oxidation of epinephrine to adrenochrome [31] at 20°. The standard reaction mixture contained epinephrine (0.3 mM), NADPH (1 mM), and 3 mg of microsomal protein in 3 ml of Tris–KCl buffer, pH 7.4.

Statistical analysis. Treated and control groups were compared with the unpaired Student's *t*-test (two-tailed); $P < 0.05$ was selected as significant. Kinetic constants were determined by Wilkinson's regression [32].

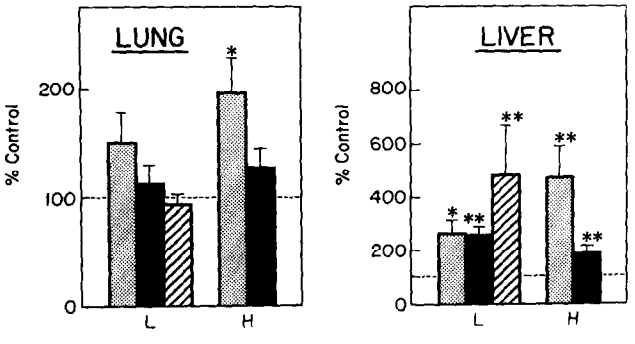

Fig. 1. Effect of *in vivo* bleomycin on lipid peroxidation in lung and liver microsomal fractions. Separate groups of rats (N = 5–6) were injected i.p. twice weekly with bleomycin, [5 units (L) or 15 units (H)] for 1.5 weeks (spotted bars), 3 weeks (solid bars) or 6 weeks (hatched bars). Controls received an equal volume of saline. Data are expressed as per cent of control rate of generation of thiobarbituric acid-reactive products. Control values were 1.34 ± 0.08 and 4.35 ± 0.57 nmoles per mg microsomal protein per hr for lung and liver fractions respectively. Key: (*) $P < 0.05$, and (**) $P < 0.01$. Data are $\bar{x} \pm$ S.E.

RESULTS

The ADP–Fe (III)-dependent generation of TBA reactive products is used commonly as an indication of peroxidizability of tissue lipids [21]. As seen in Fig. 1, there were no significant differences in the rate of ADP–Fe (III)-initiated peroxidation of lung microsomal lipids in the 1.5-, 3- or 6-week low-dose groups. In the high-dose groups, lipid peroxidation was increased to 196 per cent of control ($P < 0.05$) at 1.5 weeks and returned to normal in 3 weeks. In contrast to the lung, liver microsomal lipid peroxidation was enhanced 2- to 4-fold in all bleomycin-treated animals.

The interaction of *in vitro* addition of bleomycin with the ADP–Fe (III)-dependent lipid peroxidation system was examined in both lung and liver microsomal fractions. Bleomycin inhibited the production of thiobarbituric acid reactive products in lung and liver microsomal lipids in the standard incubation mixture which contained 12 μM Fe (III) (Fig. 2). Since lipid peroxidation is dependent upon the presence of the ADP–Fe (III)-complex in the incubation medium, the *in vitro* inhibition of lipid peroxidation may have resulted from chelation of the Fe (III). Indeed, higher concentrations of exogenous Fe (III) reversed the inhibition of lipid peroxidation in both lung and liver microsomal fractions (Fig. 2).

To circumvent the requirement for metal cofactors, soybean lipoxygenase I was used to initiate lipid peroxidation. Bleomycin had no effect on lipoxygenase-initiated formation of TBA-reactive products in lung microsomal fractions. In contrast, bleomycin caused a concentration-dependent inhibition in liver preparations (Fig. 3).

The *in vitro* generation of hydrogen peroxide by lung microsomal fractions from rats pretreated 1.5 or 3 weeks with low-dose bleomycin was decreased,

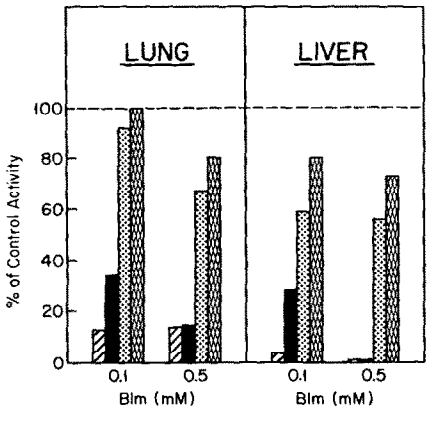

Fig. 2. *In vitro* interaction of bleomycin with ADP–Fe (III)-dependent microsomal lipid peroxidation. Lung or liver microsomal fractions (1 mg protein) were incubated with 0.5 mM NADPH, 4 mM ADP and various concentrations of FeCl (12 μM, hatched bars; 60 μM, solid bars; 120 μM, spotted bars; or 600 μM, double-hatched bars) in 0.02 M Tris–0.05 M KCl buffer, pH 7.4, at 37° for 20 min. Lipid peroxidation was estimated as thiobarbituric acid-reactive products. Data are per cent of control rate of thiobarbituric acid-reactive product formation at a specified Fe (III) concentration.

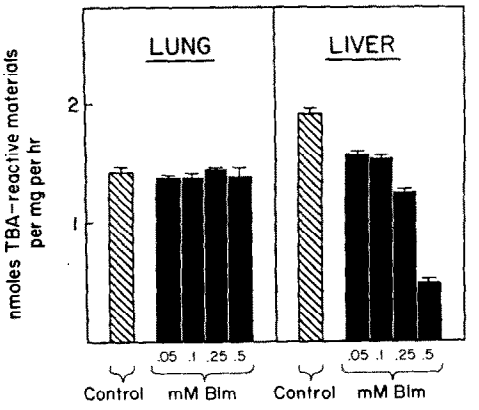

Fig. 3. Effect of bleomycin on *in vitro* microsomal lipid peroxidation initiated by soybean lipoxygenase I. Microsomal fractions (1 mg protein) were incubated with 0.1 mg of soybean lipoxygenase I in 0.02 M Tris–0.15 M KCl, pH 7.4, at 37° for 20 min. Lipid peroxidation was measured by the thiobarbituric acid method. Microsomal fractions were prepared from a pool of four to six rat lungs or livers. Data are $\bar{x} \pm$ S.D.

but in preparations dosed 6 weeks it was the same as controls (Fig. 4). High-dose bleomycin treatment suppressed lung microsomal hydrogen peroxide generation to 48 per cent of control ($P < 0.01$) at 1.5 weeks. By 3 weeks, the rate of hydrogen peroxide generation had returned to control. In contrast, the rate of hydrogen peroxide generation in liver microsomal fractions was unchanged at 1.5 weeks, but was progressively inhibited at 3 and 6 weeks in the low-dose groups and was inhibited at 3 weeks in the high-dose group.

The *in vitro* effect of bleomycin on hydrogen peroxide generation was also examined (Fig. 5). In lung preparations, a stimulation of hydrogen peroxide generation by 0.1 to 3.0 mM bleomycin was observed. In liver fractions, however, bleomycin markedly inhibited the production of hydrogen peroxide in a concentration-dependent manner, with an IC_{50} value of 2.2 mM. No effect on superoxide generation was observed in either lung or liver fractions with 0.05 to 0.5 mM bleomycin (Table 1).

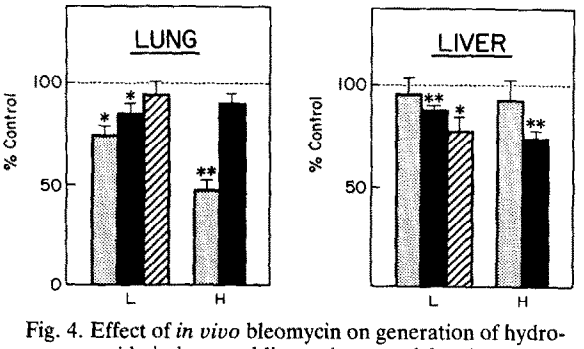

Fig. 4. Effect of *in vivo* bleomycin on generation of hydrogen peroxide in lung and liver microsomal fractions. Data are expressed as per cent of control. Details are in Fig. 1. Control rates were 200 ± 5 and 925 ± 53 nmoles formaldehyde per mg microsomal protein per hr for the lung and liver microsomal fractions respectively. Key: (★) $P < 0.05$, (★★) $P < 0.01$, N = 4–6.

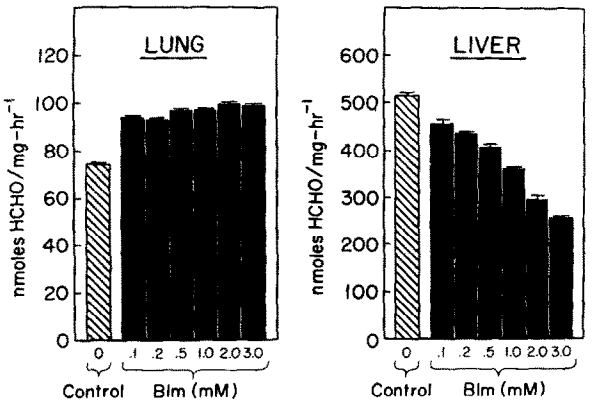

Fig. 5. Effect of bleomycin *in vitro* on hydrogen peroxide generation in lung and liver microsomal fractions. Microsomal fractions (2 mg protein) were incubated with 0.25 M methanol and 1800 units catalase in 0.02 M Tris–5 mM $MgCl_2$–0.15 M KCl buffer, pH 7.4, at 37° for 20 min in the presence of an NADPH-generating system. Formaldehyde formation was determined by the method of Nash [29]. Microsomal fractions were prepared from a pool of four to six rat lungs or livers. Data are $\bar{x} \pm$ S.D.

Bleomycin treatments *in vivo* had no effect on the cytochrome P-450-dependent *N*-demethylation of benzphetamine in lung microsomal fractions *in vitro* (Fig. 6). In contrast, the activity in liver fractions was extremely responsive to bleomycin treatments. The enzyme activity was suppressed 40 per cent as early as 1.5 weeks after treatment in both low- and high-dose groups. The inhibition persisted unchanged through the 6 weeks of low-dose treatment and became more severe at 3 weeks of high-dose treatments.

In vitro, bleomycin produced a slight stimulation ($P < 0.05$) of benzphetamine *N*-demethylase activity in lung fractions (Fig. 7). In liver preparations, an inhibition of this enzyme activity was observed at 0.5 mM bleomycin. Kinetic analysis indicated that bleomycin inhibits hepatic benzphetamine *N*-demethylase activity in a noncompetitive manner with a K_i of 1 mM. The *N*-demethylation of ethylmorphine, another type I substrate of the hepatic mixed function oxidase system, was also inhibited in liver microsomal fractions following *in vivo* or *in vitro* bleomycin (data not shown).

DISCUSSION

Lipid peroxidation is widely regarded to be a fundamental process in the development of some forms of tissue damage. In addition to the oxidative destruction of membrane lipids, structural damage to membrane-bound enzymes may also result from this process. Recently, evidence has been presented that both superoxide and hydroxyl radicals may initiate lipid peroxidation [18, 19]. Bleomycin may generate superoxide anion radicals from molecular oxygen in the presence of Fe (II) [15]. Simultaneous formation of the highly reactive hydroxyl radical has been demonstrated indirectly by spin trapping techniques [16, 17]. Thus, an enhancement of ADP–Fe (III)-initiated lipid peroxidation by bleomycin in microsomal fractions of lung, the target organ for toxicity, was anticipated. However, the sensitivity of lung microsomal lipids to peroxidation after low doses of bleomycin was not increased. The observed 2-fold stimulation following 1.5 weeks of high-dose treatment indicates that very high doses of bleomycin are necessary to augment ADP–Fe (III)-initiated lipid peroxidation. It is surprising that the liver, which has a high level of bleomycin inactivating enzyme and does not demonstrate toxicity from bleomycin, exhibited markedly increased susceptibility to lipid peroxidation, whereas the lung, which is deficient in the inactivating enzyme [33], was comparatively unaffected. It is possible that only a small population of lung cells or only a few cell types are susceptible to damage by bleomycin. A significant change in lipid peroxidation susceptibility in these damaged cells would be difficult to detect. The stimulation of lipid peroxidation observed in the 1.5-week high-dose bleomycin group may indicate damage to

Table 1. Effect of bleomycin on superoxide generation from lung and liver microsomal fractions*

	Increase in optical density units per min at 480 nm absorbance
Lung	
Control	0.0037
+Bleomycin (5 μM)	0.0038
+Bleomycin (50 μM)	0.0036
+Bleomycin (500 μM)	0.0038
Liver	
Control	0.0016
+Bleomycin (5 μM)	0.0014
+Bleomycin (50 μM)	0.0015
+Bleomycin (500 μM)	0.0014

* The standard reaction mixture contained epinephrine (0.3 mM) NADPH (1 mM) and 3 mg of microsomal protein in 3 ml of 0.02 M Tris–0.15 M KCl buffer, pH 7.4, with the presence of various concentrations of bleomycin. The rate of epinephrine oxidation to adrenochrome was monitored by the increase in absorbancy at 480 nm as described by Misra and Fridovich [31].

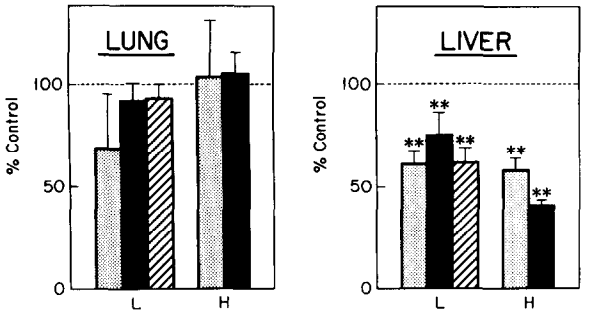

Fig. 6. Effect of *in vivo* bleomycin on benzphetamine *N*-demethylase activity in lung and liver microsomal fractions. Data are expressed as per cent of control rates of metabolism (42 ± 2 and 311 ± 18 nmoles of formaldehyde formed per mg microsomal protein per hr for lung and liver respectively). Key: (★★) $P < 0.01$, N = 4–6. Data are $\bar{x}$ ± S.E. L = low doses of bleomycin (5 units) and H = high doses (15 units) for 1.5 weeks (spotted bars), 3 weeks (solid bars) or 6 weeks (hatched bars).

many cell types. On the other hand, in the liver, which is primarily composed of parenchymal cells, increased ADP–Fe (III)-initiated lipid peroxidation was prominent in microsomal fractions from all bleomycin-treated animals.

Biochemical quantification of lipid peroxidation is complicated by the difficulty in identifying reaction products. Fong *et al.* [34] have proposed that lipid peroxidation may be initiated by hydroxyl radicals generated from superoxide in the presence of a chelated ADP–Fe (III) complex. However, in both lung and liver microsomal fractions, bleomycin inhibited ADP–Fe (III)-dependent lipid peroxidation and had no stimulative effect on superoxide production. Reversal of the bleomycin inhibition by addition of Fe (III) suggests that the inhibition was an artifact resulting from chelation of Fe (III) by bleomycin. Yamanaka *et al.* [35], however, observed an inhibition of liver microsomal lipid peroxidation by bleomycin in the absence of catalytic ADP–Fe (III). This agrees with our observation of bleomycin inhibition of liver microsomal lipid peroxidation when peroxidation was initiated by soybean lipoxygenase I. The ineffectiveness of bleomycin in stimulating lipoxygenase-initiated lipid peroxidation in lung microsomal fractions further confirms the insensitivity of the bleomycin-treated lung to lipid peroxidation. These results suggest that bleomycin pulmonary toxicity is not directly related to lipid peroxidation and activated oxygen damage.

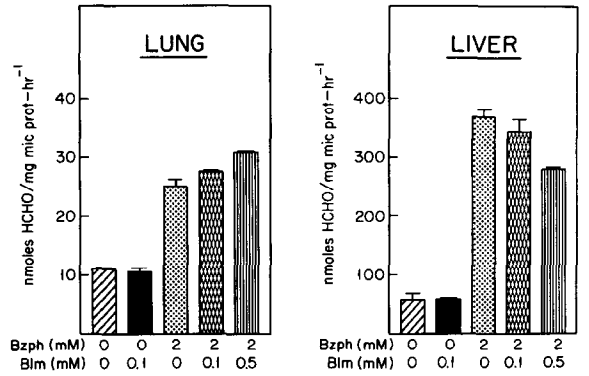

Fig. 7. Effect of bleomycin *in vitro* on benzphetamine *N*-demethylase activity in lung and liver microsomal fractions. Microsomal fractions (1 mg protein) were incubated with 2 mM benzphetamine hydrochloride in 0.02 M Tris–5 mM $MgCl_2$–0.15 M KCl buffer, pH 7.4, at 37° for 20 min in the presence of an NADPH-generating system. Formaldehyde formation was measured by the method of Nash [29]. Microsomal fractions were prepared from a pool of four to six rat lungs or livers. Data are $\bar{x}$ ± S.D.

Generation of hydroxyl radicals and singlet oxygen requires hydrogen peroxide, superoxide, and a metal catalyst [36, 37]. In microsomal fractions, hydrogen peroxide is generated from the dissociation of oxycytochrome P-450, peroxycytochrome P-450, and autooxidation of reduced flavoproteins [38]. The concentration-dependent inhibition of hydrogen peroxide generation by bleomycin in liver microsomal fraction suggests that the mixed function oxidase system may be affected. Inhibition of the cytochrome P-450-dependent *N*-demethylations of benzphetamine and ethylmorphine further suggests that bleomycin disrupts hepatic mixed function oxidation. The noncompetitive kinetics of the bleomycin inhibition indicates that bleomycin is not an alternate substrate. Since bleomycin is a potent chelating agent for metals [39], inhibition of this hemoprotein enzyme system could be due to chelation. Several chelating agents, such as 2,2′-bipyridine and 1,10-phenanthroline, inhibit the hepatic mixed function oxidase system [40, 41]. Loss of drug-metabilizing activity due to cytochrome destruction as a result of lipid peroxidation has been well documented [23, 24]. Our observation of suppression of hepatic cytochrome P-450-dependent enzyme activities, hydrogen peroxide generation, and stimulation of lipid peroxidation in bleomycin-treated animals suggests that disruption of the endoplasmic reticulum and/or the cytochrome P-450 itself may be involved.

The site of microsomal superoxide generation is the flavoprotein NADPH-cytochrome P-450 reductase [42]. The lack of bleomycin effect on superoxide generation further implies that cytochrome P-450 may be the component of the mixed function oxidase system which is affected. Although hepatic toxicity of bleomycin is not commonly observed, our studies clearly suggest that repeated i.p. bleomycin treatments may impair xenobiotic metabolism. Such impairment may be related to the high bleomycin level attained in hepatic tissues after i.p. administration [43]. The same enzymatic systems in the lung are resistant to effects of bleomycin.

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