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PHARMACOLOGICAL MODULATION OF THE PNEUMOTOXICITY OF 3-METHYLINDOLE

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Abstract—The nature of the reactive metabolite of 3-methylindole is investigated using microsomal preparations prepared from the lungs of cattle. Nucleophilic thiol agents, glutathione, L-cysteine and *N*-acetyl-L-cysteine, protected microsomal proteins against alkylation by the reactive metabolite of 3-methylindole. The cytosol fraction from the lungs of cattle increased the protective effect of these thiol agents. Pretreatment of sheep with diethylmaleate, which depletes glutathione, increased the severity of the pneumotoxic effect of 3-methylindole, whereas pretreatment with L-cysteine decreased the severity of this effect. These findings are consistent with a hypothesis that an electrophilic reactive metabolite of 3-methylindole is responsible for its pneumotoxic effect and implies that glutathione and glutathione *S*-transferases are involved in the detoxification of this reactive metabolite. Nucleophilic thiol agents can be useful in the prevention of reactive metabolite induced-lung injury.

3-Methylindole (skatole) (3MI) is present in cigarette smoke in substantial amounts [1] and is produced in the rumen of cattle [2] and in the lower gut of man and animal. There is considerable evidence that 3MI, produced from other indolic substrates in the rumen, is the cause of naturally occurring Acute Bovine Pulmonary Emphysema ("fog fever") [3, 4], an important respiratory disease of adult grazing cattle. The possible implication of 3MI in the aetiology of pulmonary disease in man has been suggested [5].

Oral and intravenous administration of 3MI causes severe pulmonary lesions in cattle, sheep and goats [6–9]. We have investigated the mechanism of the toxic action of 3MI towards the lung and in a previous communication we have suggested that the 3MI-induced pulmonary damage is due to a cytotoxic alkylating metabolite and we have shown that 3MI undergoes metabolic activation by a cytochrome P-450 dependent mixed function oxidase to a reactive metabolite which becomes covalently bound to cellular macromolecules of the lung *in vivo* and *in vitro* [10]. In this present work the nature and detoxifying pathways of the reactive metabolite of 3MI and the possible pharmacological modulation of the 3MI-pneumotoxicity are investigated.

MATERIALS AND METHODS

Chemicals. [G-^{3}H]-3MI (131 μCi/mmole) was prepared from L-[G-^{3}H]tryptophan as described previously [10]. Diethylmaleate was obtained from B.D.H. Chemicals Ltd. (Poole, U.K.). L-Cysteine was obtained from Koch-Light Ltd. (Colnbrook, U.K.). *N*-Acetyl-L-cysteine and glutathione (GSH) were obtained from Sigma London Chemical Co. Ltd. (Poole, U.K.). Reduced nicotinamide adenine dinucleotide phosphate (NADPH), glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Boehringer Corporation (Mannheim, West Germany). "Cremophor-El" was obtained as a gift from Victor Blagdon & Co. (Croydon, U.K.).

Preparation of washed microsomes. Lung tissues from five 6–12 month old Ayrshire breed calves were used in each experiment. Preparation of microsomes from the lung of calves was carried out, in ice-cold 0.154 M KCl and 0.05 M Tris–HCl buffer (pH 7.4), as described previously [10]. The 105,000 *g* supernatant (cytosol fraction) was saved. Microsomal pellets were suspended in phosphate buffer (0.1 M, pH 7.4) and resedimented by ultracentrifugation at 105,000 *g* for 50 min. The resultant washed microsomal pellets were rehomogenized in phosphate buffer (0.1 M, pH 7.4). Protein concentration in the microsomal suspension was measured according to the procedure of Lowry *et al.* [11].

Preparation of pulmonary cytosol fraction. Twenty ml of the first 105,000 *g* supernatant of the lung of calves was dialyzed against 5 l of 0.154 M KCl and 0.05 M Tris–HCl buffer pH 7.4 (five changes one litre each) for 18 hr at 4° to eliminate endogenous glutathione present in the cytosol fraction. Protein concentration was determined in the cytosol fraction [11].

Effect of various concentrations of glutathione on the covalent binding of 3MI-metabolite to microsomal proteins. One ml phosphate buffer (pH 7.4, 0.1 M) containing washed lung microsomes (5 mg protein), a NADPH generating system composed of 8.5 mg NADPH, 63 mg glucose-6-phosphate and 2 units glucose-6-phosphate dehydrogenase, 0.25 μmole [G-^{3}H]-3MI (131 μCi/mmole) and the appropriate amount of GSH was incubated at 37° for 30 min. Incubations were run in duplicate. Covalently bound [G-^{3}H]-3MI metabolites [10] and protein concentrations were determined [11].

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Effect of the cytosol fraction and nucleophilic agents glutathione, L-cysteine and N-acetyl-L-cysteine on the covalent binding of 3MI-metabolite to microsomal protein. Duplicate incubations were used; each contained a total volume of 1 ml phosphate buffer (0.1 M, pH 7.4) containing lung microsomes (5 mg protein), unboiled or boiled (100° for 10 min) lung cytosol (1.5 mg protein), the appropriate amount of GSH, cysteine or *N*-acetyl-L-cysteine, if required, an NADPH generating system as described above and 0.25 μmole [G-^{3}H]-3MI (131 μCi/mmole). Incubations were run for 20 min at 37°. Assay for covalently bound 3MI-metabolite and proteins was carried out as described before.

Effect of diethylmaleate and L-cysteine on the 3MI-pneumotoxicity. Male, castrated, Blackface cross-bred sheep (38–55 kg body wt) were used in this experiment. All compounds were administered by intraperitoneal injection. 3MI solution (30 mg/ml) was prepared in 10% (v/v) "Cremophor-EL" in physiological saline solution. (3MI is poorly soluble in saline; "Cremophor-EL" is a water-miscible oil.) L-Cysteine (100 mg/ml) was dissolved in physiological saline solution immediately before use and was administered 60 min before 3MI. Diethylmaleate was administered without dilution 30 min before 3MI administration. Sheep were given either 3MI alone or 3MI after pretreatment with diethylmaleate or L-cysteine. The severity of the pulmonary toxicity was assessed by (1) observing animals after 3MI administration; (2) determining the time of death of sheep; and (3) determining the degree of pulmonary oedema post-mortem. This may be done by two methods: (a) determining the lung weight as a percentage of total body weight, and (b) determining the ratio of wet lung tissue wt/dry lung tissue wt. This was determined by taking six pieces of lung tissue (a total of 2–3 g) randomly from different sites from both lungs in a tared porcelain dish. Wet tissue wt was determined and tissue left to dry to a constant weight in an oven at 65°; (4) histopathological examination: portions of tissue were removed from the lung and bronchial tree, immediately after death or euthanasia, fixed in 10% buffered saline formalin solution, dehydrated and embedded in paraffin. Sections (5 μm thick) were stained with hematoxylin and eosin.

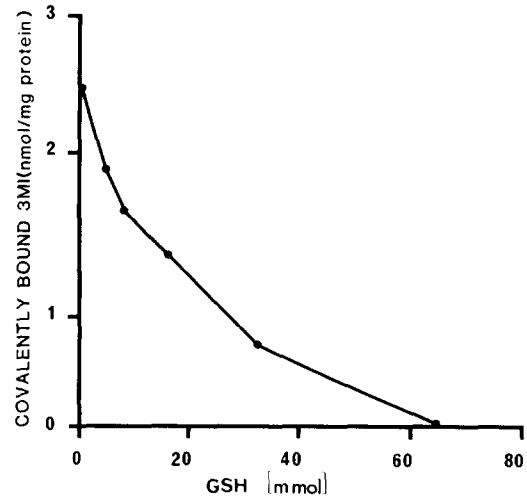


Fig. 1. The extent of covalent binding of [G-^{3}H]-3MI metabolites to lung microsomes in the presence of different concentrations of GSH.

RESULTS

Effect of glutathione on the covalent binding of [G-^{3}H]-3MI to microsomal proteins

GSH caused a concentration dependent inhibition of covalent binding of 3MI-metabolites to microsomal protein (Fig. 1). A concentration of 64 mmoles GSH caused almost complete inhibition (99%) of covalent binding.

Effect of the cytosol fraction and nucleophilic thiol compounds on the covalent binding of [G-^{3}H]-3MI-metabolites to microsomal protein

Addition of unboiled cytosol alone but not boiled cytosol fraction inhibited covalent binding to microsomal proteins (Fig. 2). Addition of increasing con-

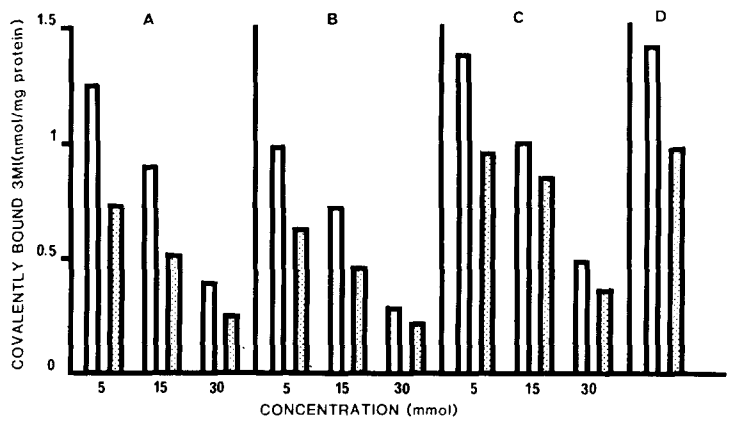


Fig. 2. Effect of cytosol and three concentrations (5, 15 and 30 mmoles) of glutathione (A), L-cysteine (B) and *N*-acetyl-L-cysteine (C) on the covalent binding of [G-^{3}H]-3MI. Incubations (D) contained none of these compounds. Stippled bars represents incubations containing unboiled lung cytosol. Open bars represents incubations containing boiled lung cytosol.

Table 1. Effect of 3-methylindole on normal sheep and on sheep pretreated with diethylmaleate or L-cysteine

Group	Animal no.	Treatment	Weight (kg)	Time of death	Lung weight (% body weight)	Wet lung tissue weight / Dry lung tissue weight
I	1	3MI	38	S	N.D.	N.D.
	2	(0.2 g/kg)	40	S, K, 7 d	1.5	4.9
	3		40	S, K, 7 d	1.3	5.1
	4		45	S, K, 72 hr	1.5	4.8
	Mean*				1.4 ± 0.1	4.9 ± 0.1
II	5	3MI	45	D, 18 hr	N.D.	N.D.
	6	(0.2 g/kg)	43	D, 20 hr	N.D.	N.D.
	7	+	45	D, 7 hr	1.8	5.3
	8	DM	50	D, 15 hr	1.4	5.1
	9	(0.15 ml/kg)	40	D, 8 hr	1.5	5.4
	Mean				1.6 ± 0.1	5.3 ± 0.1
III	10	3MI	45	D, 2 hr	N.D.	N.D.
	11	(0.3 g/kg)	43	D, 7 hr	1.8	7.5
	12		55	D, 5 hr	2.0	5.8
	13		45	D, 10 hr	1.3	5.1
	Mean				1.7 ± 0.3	6.1 ± 0.9
IV	14	3MI	55	D, 18 hr	N.D.	N.D.
	15	(0.3 g/kg)	40	D, 20 hr	N.D.	N.D.
	16	+	45	S, K, 4 d	1.6	4.6
	17	L-cyst. (0.3 g/kg)	43	S, K, 4 d	1.1	4.9
	Mean				1.5 ± 0.4	4.7 ± 0.2
V	18	DM	44	S	N.D.	N.D.
	19	(0.15 ml/kg)	43	S	N.D.	N.D.
VI	20	L-cyst.	40	S	N.D.	N.D.
	21	(0.3 g/kg)	42	S	N.D.	N.D.

* Mean ± S.E.M.

3MI = 3-methylindole; DM = diethylmaleate; L-cyst. = L-cysteine; S, K = survived, and killed by pentobarbitone euthanasia; D = died; N.D. = not determined.

centrations of GSH, L-cysteine or *N*-acetyl-L-cysteine caused a concentration-dependent inhibition of covalent binding to microsomal proteins. Addition of unboiled cytosol together with GSH or L-cysteine or *N*-acetyl-L-cysteine caused a greater degree of inhibition of covalent binding. The difference between boiled and unboiled cytosol in inhibiting covalent binding was greater with GSH than with L-cysteine or *N*-acetyl-L-cysteine.

Effect of diethylmaleate and L-cysteine on the 3MI-pneumotoxicity

Time of death and measurements of oedema are summarized in Table 1.

Administration of 3MI alone, or after pretreatments used in this experiment, caused respiratory distress in sheep. This was manifested by central depression, nasal discharge and tachypnoea within 10 to 20 min after 3MI administration. As respiratory distress progressed animals showed difficulty breathing and a decrease in respiratory rate. Audible expiratory grunt was very pronounced in animals dying after 3MI administration. These animals assumed lateral recumbency before death.

Administration of 3MI (0.2 g/kg) alone (group I) did not cause death in any of the four treated sheep. All sheep in this group showed signs of respiratory distress which was maximal at 2 hr after 3 MI administration. Animals improved over the next 10 hr and became apparently normal by 72 hr. Histopathological examination of lungs of these animals showed that changes in alveolar morphology were mild and focal in distribution. Some areas were normal whereas in other areas alveolar septa were thickened due to oedema and infiltration with mononucleated cells. The epithelial lining of the bronchi was normal and their lumen contained mononucleated cells whose cytoplasm had a foamy appearance and neutrophils mixed with cell debris and acidophilic material. In contrast pretreatment of sheep with diethylmaleate before administration of the same dose of 3MI (0.2 g/kg) (group II) increased the severity of respiratory distress and pathological changes in the lung leading to death of all five treated sheep. In these sheep respiratory distress was progressive. About 4 hr after 3MI administration all animals assumed lateral recumbency with extended neck and died. Histopathological changes in the lungs of these animals were generally severe except for small foci showing mild changes in alveolar morphology. Severe congestion and foci of recent haemorrhages were observed. The alveoli contained acidophilic deposit and mononucleated cells and alveolar septa were thickened. The epithelial lining of bronchi was apparently normal and their lumens contained considerable numbers of cells (Fig. 3(b)). These cells

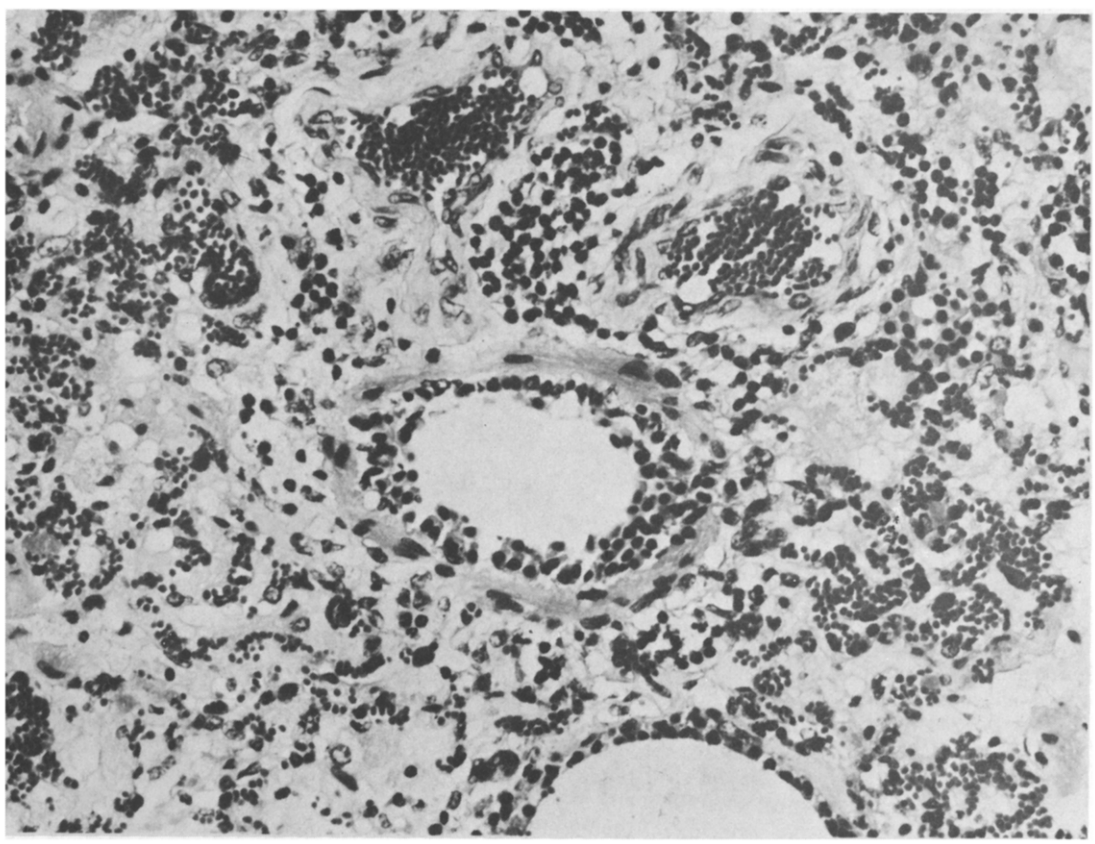
were exfoliated epithelial cells, they had columnar, cuboidal or rounded appearance, pyknotic nuclei and some of them had cilia (Fig. 3(c)). The epithelial cells lining the bronchioles had pyknotic nuclei and were seen in different degrees of detachment from the basement membrane (Fig. 3(a)). Some bronchioles had few detached cells, whereas the lining of others was completely denuded. The initial dose used of diethylmaleate (0.6 ml/kg) caused severe depression and muscle trembling in two treated sheep. One of these two sheep died at about 24 hr and the other was in poor condition when killed at 48 hr. A smaller dose of diethylmaleate (0.15 ml/kg) did not cause any observable toxic symptoms in two sheep. These were in good condition and maintained good appetite after diethylmaleate administration (group V).

A larger dose of 3MI (0.3 g/kg) (group III) caused progressive respiratory distress and histopathological changes similar to those described for group II leading to death of all (four) treated sheep within 10 hr after 3MI administration. When the same dose of 3MI (0.3 g/kg) was administered to four sheep pretreated with L-cysteine (group IV), two sheep showed toxicity symptoms and had histopathological changes similar to those described for group I and survived, while the other two sheep developed ruminal bloat and died. Death of these two sheep was delayed (18–20 hr) after 3MI administration.

Administration of L-cysteine (0.3 g/kg) alone (group VI) caused slight ruminal bloat in one of two treated sheep. This bloat was observed 2 hr after L-cysteine administration and lessened during the next 6 hr. The other animal did not show any sign of abnormality. Both animals maintained good appetite and were in good condition.

DISCUSSION

Strong nucleophilic thiol compounds GSH, L-cysteine and *N*-acetyl-L-cysteine inhibited covalent binding of the reactive metabolite of 3MI to microsomal proteins suggesting that this reactive metabolite is a highly electrophilic species. Low molecular weight nucleophilic agents inhibit covalent binding to microsomal proteins by providing alternative nucleophilic sites for covalent binding. It is well known that the conjugation of GSH with foreign compounds and their metabolites which are sufficiently electrophilic can occur spontaneously (non-enzymatically). Also a group of enzymes (glutathione-*S*-transferases) present in the cytosol (soluble) fraction of cells can catalyze the conjugation of GSH with a wide variety of chemical com-



(a)

Fig. 3. Photomicrograph showing bronchiolar necrosis in the lungs of sheep which died after receiving doses of 3MI (0.2 g/kg) and diethylmaleate (0.15 ml/kg). (a) The epithelial lining of bronchioles seen detached from basement membrane and with pyknotic nuclei. (b) A bronchus with normal epithelial lining and its lumen containing exfoliated epithelial cells. (c) Higher magnification of cells in the lumen of the bronchus in (b). Cells had columnar or cuboidal appearance and pyknotic nuclei and some of them were ciliated. (H & E stain; magnification: (a) ×250, (b) ×35 and (c) ×1300).

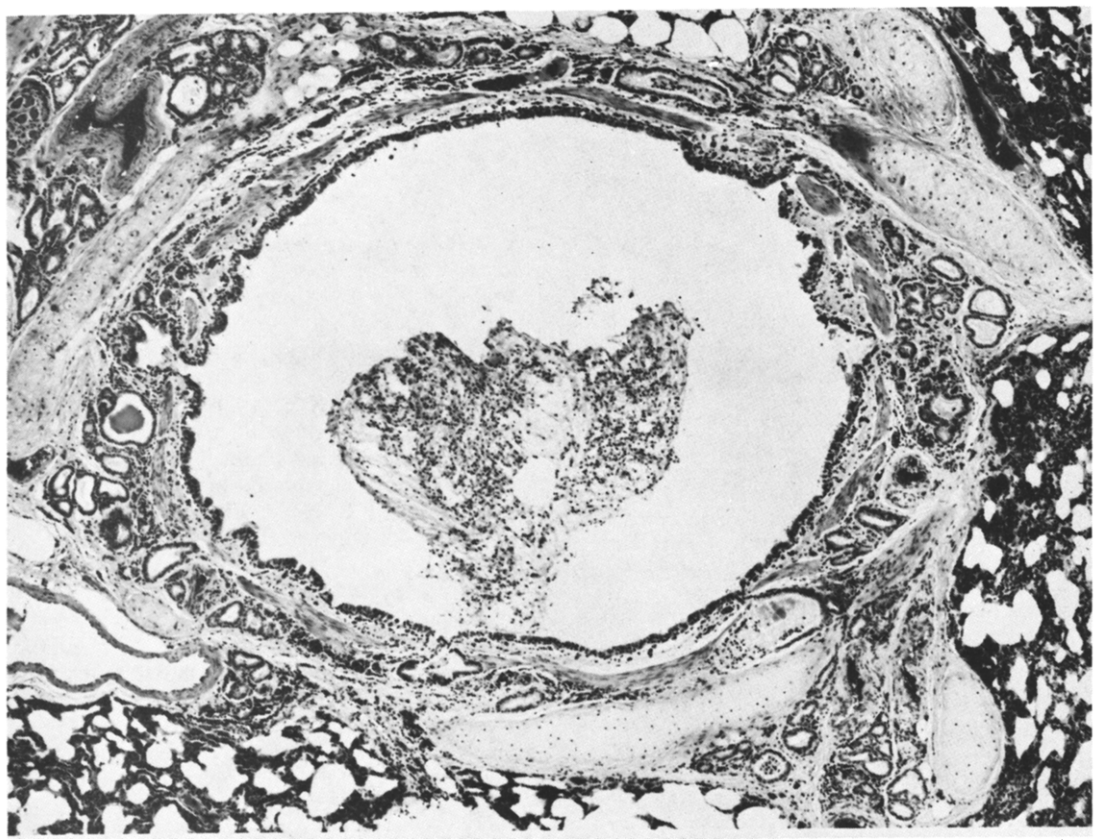


Fig. 3(b)

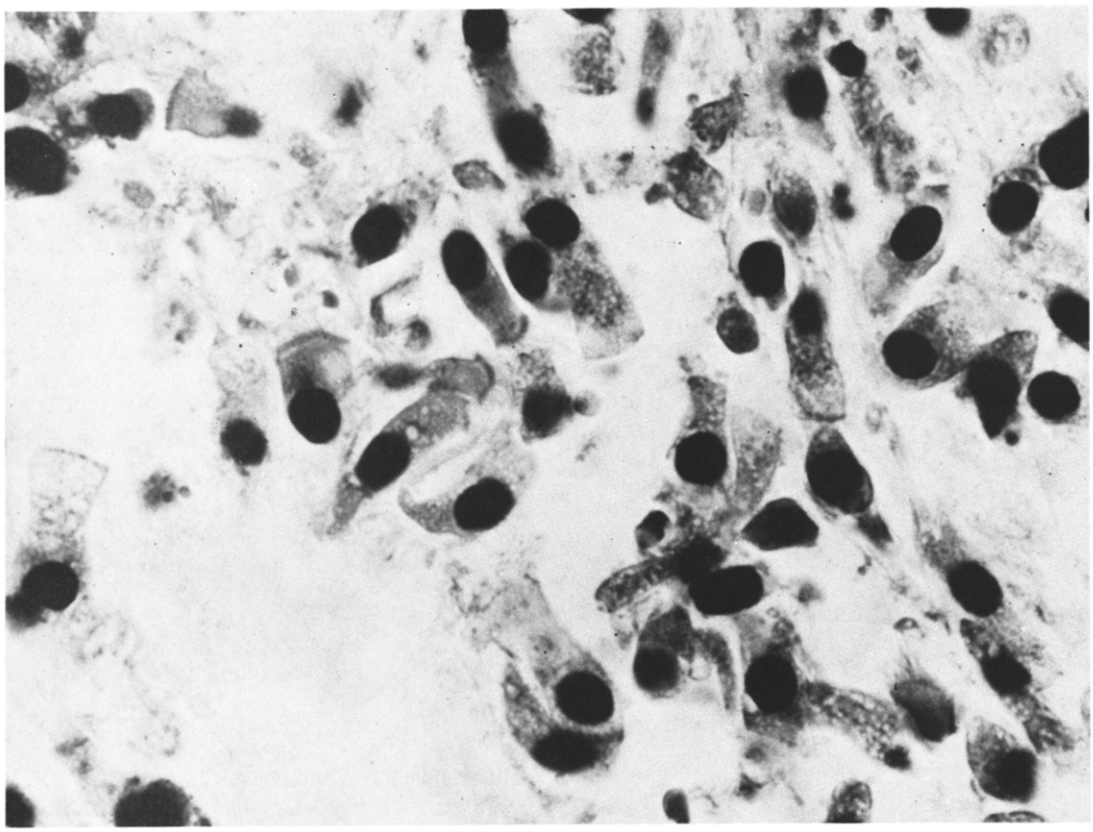


Fig. 3(c)

pounds [12–17]. Results of this present study have shown that GSH inhibited covalent binding of 3MI to microsomal proteins. Addition of the cytosol fraction caused an increase in the inhibitory effect of GSH. These findings indicate that the reaction between GSH and the reactive metabolite of 3MI can occur spontaneously in the absence of cytosolic enzymes and also that cytosolic enzymes can catalyze this reaction. The effect of lung cytosol fraction in decreasing covalent binding of 3MI metabolite to microsomal proteins was greatest when the cytosol fraction was used with GSH compared to L-cysteine or *N*-acetyl-L-cysteine. Similar observations were reported by Rollins and Buckpitt [16] while studying the role of liver cytosol in catalyzing the conjugation of reduced GSH with a reactive metabolite of the hepatotoxic drug paracetamol. Furthermore their studies [16] have shown that these cytosolic enzymes appear to play a major role in facilitating the conjugation of the reactive metabolite of paracetamol with GSH when the concentration of GSH is low. In view of these findings suggesting that GSH is important in the inactivation of the reactive metabolite of 3MI, it was interesting to study the effect of pretreatments which are known to alter the availability of GSH on the severity of the 3MI-induced pneumotoxicity. It is known that diethylmaleate depletes GSH in many organs including the lung [18, 19]. The dose range and effects of diethylmaleate in sheep have not been investigated previously. The dose used initially (0.6 ml/kg) is equal to that used in rats by Boyd and Burka [20] to deplete lung GSH but in this present work this dose caused severe toxic effects in sheep. The smaller dose of diethylmaleate (0.15 ml/kg) (group V) did not cause signs of toxicity in sheep. Pretreatment with diethylmaleate increased the severity of the pneumotoxic effects of 3MI in sheep. Conversely L-cysteine protected sheep against the pneumotoxic effect of 3MI. The protective effect of L-cysteine against 3MI can be attributed to at least two factors: (1) increased availability of GSH offering alternative nucleophilic sites for covalent binding with the reactive metabolites of 3MI, thus protecting cellular macromolecules from alkylation by the reactive metabolites; and (2) L-cysteine itself can act as a nucleophilic substrate for covalent binding with the 3MI reactive metabolites.

These findings are consistent with a theory that an electrophilic reactive metabolite of 3MI is formed *in vivo* and that this reactive metabolite is responsible for the 3MI-induced pulmonary damage, and strongly suggest that GSH and glutathione-*S*-transferases play an important role in the detoxification of 3MI reactive metabolites. In favour of this hypothesis: GSH protected microsomal proteins, *in vitro*, from alkylation by the reactive metabolite of 3MI and glutathione-*S*-transferase (cytosol fraction) increased the protective effect of GSH (*in vitro*). Depletion of GSH increased, while L-cysteine, a precursor of GSH, decreased the severity of the 3MI induced pulmonary damage *in vivo*. 3-Methyloxindole has been shown to be the major metabolite of 3MI in cattle [4] and has been shown to be without toxicity to the lung when infused into anaesthetised calves, whereas in the same model 3MI is extremely toxic. It is probable that the electrophilic reactive metabolite is intermediate between 3MI and 3-methyloxindole and is an epoxide [10].

While the role of GSH and glutathione-*S*-transferase in the inactivation of hepatotoxic and carcinogenic foreign compounds is well documented [12–16] little is known about the importance of this detoxifying pathway in protecting the lung against reactive metabolite-induced injury. Other investigators [20] have shown that pretreatment of rats with diethylmaleate increases both the alkylation of lung tissue and severity of lung lesions caused by 4-ipomeanol, a compound which forms reactive metabolites toxic to the lung, suggesting that GSH is involved in the inactivation of the reactive metabolites of this compound. This has been confirmed by the isolation of two glutathione-4-ipomeanol metabolite conjugates from an incubation mixture containing lung or liver microsomes, NADPH, [^{3}H]-4-ipomeanol and GSH. Addition of lung or liver cytosol did not enhance the formation of these conjugates suggesting that cytosolic glutathione-*S*-transferases are not involved in the inactivation of the 4-ipomeanol reactive metabolites [17].

Histopathological studies reported here and those by other investigators [9] have shown that necrosis of bronchiolar epithelium is a reproducible and consistent effect of lethal doses of 3MI. Changes in bronchiolar morphology were consistent in different areas of the lung of the same animal and were similar in different animals which had received the same treatment. In contrast changes in alveolar morphology varied in severity in different areas of the lung of the same animal. Similarly pneumotoxic furano compounds and halobenzenes produced consistent changes in the bronchiolar epithelium, whereas the changes in alveolar morphology were less consistent [20–23]. It has been suggested that the Clara cells are the site of cytochrome P-450 dependent mixed function oxidase in the lung. This hypothesis is based on findings showing that Clara cells are the target for reactive metabolite-forming pneumotoxic compounds whose reactive metabolites are formed by a cytochrome P-450-dependent mechanism [22, 24]. Presence of large numbers of Clara cells in the epithelium of bronchioles renders these bronchioles more susceptible to the effect of these pneumotoxic compounds. Oedema and alteration in alveolar morphology are changes secondary to bronchiolar injury.

Rapid intravenous injection of 3MI induces an anaphylactoid-like reaction in calves, which can be alleviated by antagonists to known mediators of anaphylaxis [25]. Based on these observations it has been suggested that 3MI causes pulmonary lesions through the release of mediators of anaphylaxis.

The pathogenesis of the 3MI-induced lung lesions would appear to start with specific cellular injury caused by the reactive metabolite of 3MI. Cellular injury can stimulate the release of mediators of anaphylaxis causing the immediate anaphylactoid-like reaction recorded by Atkinson *et al.* [25] and can stimulate inflammatory changes in the lung. Therefore, although antagonists of mediators of anaphylaxis have been reported to alleviate the immediate anaphylactoid-like reaction following administration of 3MI, these antagonists did not alter the severity of 3MI induced lung lesions [26]. This

hypothesis is also consistent with the sequence of ultrastructural changes showing that signs of cellular injury occur as early as two hours and precede the development of pulmonary oedema after oral administration of 3MI in goats, suggesting that the pulmonary oedema is a part of an inflammatory response to specific cellular injury [27]. Furthermore, *in vitro* studies [4] have shown that 3MI does not cause contraction of the pulmonary vein. Therefore hydrodynamic imbalance across the alveolo–capillary membrane caused by pulmonary venoconstriction cannot be considered as the initial step in the pathogenesis of 3MI-induced lung injury as has been suggested by other investigators [28].

Results of the study have shown that nucleophilic thiol compounds are worthy of study for the treatment of 'fog fever' in cattle. Administration of these compounds is the only method of treatment of toxicity with the reactive metabolite-forming hepatotoxic drug paracetamol [29, 30]. In the treatment of paracetamol poisoning the time of administration and bioavailability of the administered thiol compound is critical for the effectiveness of treatment and treatment is of doubtful value once the disease has progressed.

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