THE ROLE OF 3-METHYLINDOLE IN THE AETIOLOGY OF "FOG FEVER" IN CATTLE

by

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CONTENTS

SUMMARY

SUMMARY

3-Methylindole (skatole) is a metabolite of L-tryptophan formed in the rumen of cattle. There is considerable evidence that 3-methylindole is the cause of fog fever (acute bovine pulmonary emphysema) in cattle. Experimentally, administration of 3-methylindole causes acute pulmonary oedema and emphysema in cattle, goat and sheep. The aim of this study was to investigate the pharmacokinetics and mechanism of action of 3-methylindole with special reference to fog fever, the most important respiratory disease problem of grazing adult cattle in Britain.

Samples of ruminal fluid taken from different animals and from the same animal on different days showed considerable variations in their ability to metabolize L-tryptophan to 3-methylindole (8-78% conversion of L-tryptophan to 3-methylindole, during 24 hours incubation). These variations provided an explanation for the considerable individual variations in the severity of respiratory distress seen in cattle after oral administration of L-tryptophan and observed after sudden change to better grazing. It was suggested that the increased rate of conversion of L-tryptophan to 3-methylindole, rather than the excessive intake of the amino acid may be responsible for the production of the disease.

Certain carbohydrates (fructose, glucose, lactose, galactose, sucrose, mannitol, starch and inulin), substances related to carbohydrates (citrate, lactate, acetate and glycerin) and antibacterial agents (ampicillin, penicillin, streptomycin, tetracycline, chloram-

phenicol, sulfaguanidine and sulfamethoxypyridazine) inhibited, to different degrees, the conversion of L-tryptophan to 3-methylindole.

An oral dose of L-tryptophan (0.5 g/kg body weight) did not produce respiratory distress in any of four treated adult cattle. Failure of L-tryptophan to produce the disease was attributed to failure of conversion of the amino acid to 3-methylindole which was not detected in plasma of the treated cattle. The absorption of 3-methylindole, after oral administration in cattle, is rapid. 3-Methyloxindole is a metabolite of 3-methylindole in cattle. Mean plasma half lives of 3-methyloxindole, 3-methylindole and 3-methylindole metabolites were estimated to be 11, 16 and 48 minutes respectively. It was concluded that measurement of the concentration of 3-methylindole or 3-methylindole metabolites in single plasma samples would not reflect the magnitude of production of 3-methylindole in the rumen of cattle.

3-Methylindole (40 µg/ml) and 3-methyloxindole (50 µg/ml) did not cause the release of mediators of anaphylaxis from chopped bovine lung preparations (in vitro). 3-Methylindole and 3-methylindole analogues (3-methyloxindole, 3-phenylindole, 5-methylindole and 7-methylindole) (5-640 µg/ml) did not cause contraction of the isolated pulmonary artery, pulmonary vein, trachea or bronchus of calves. It was concluded that the initial step in the pathogenesis of the 3-methylindole-induced pulmonary damage does not involve an action on the pulmonary vein or the release of mediators of anaphylaxis in cattle.

Radioactivity from tritiated 3-methylindole became covalently

bound to tissues of several organs when tritiated 3-methylindole was administered, intravenously, to calves. Lung tissue showed the highest concentration of covalently bound metabolites. In vitro studies showed that this covalent binding is catalyzed by a microsomal enzyme system with the classical characteristics of a cytochrome P-450 dependent mixed function oxidase. This enzyme system activates 3-methylindole to a chemically reactive highly electrophilic metabolite which becomes covalently bound to nucleophilic sites on cellular macromolecules. The 3-methylindole-reactive metabolite can be detoxified by spontaneous and glutathione-S-transferase-catalyzed conjugation with glutathione. Pretreatment of sheep with diethyl-maleate (depletes glutathione) and L-cysteine (increases glutathione) was shown to increase and decrease, respectively, the severity of pneumotoxic effect of 3-methylindole.

3-Methyloxindole did not become covalently bound to microsomal proteins, in vitro, and did not cause acute pulmonary toxic effects similar to those produced by 3-methylindole, in vivo, suggesting that the 3-methylindole-reactive metabolite is intermediate between 3-methylindole and 3-methyloxindole.

The liver possess an enzyme system capable of catalyzing the covalent binding of 3-methylindole metabolites to microsomal proteins, in vitro. This enzyme system was qualitatively identical to that of the lung. The Michaelis constants of the lung and liver systems were 0.37 and 0.44 mmol 3-methylindole and maximal velocities of microsomal alkylation were 100 and 556 pmol covalently bound 3-methylindole/mg microsomal protein/minute, respectively. These findings suggest that the specificity of 3-methylindole towards

the lung is not due to higher rate of reactive metabolite formation in the lung, but is probably due to deficiency of the lung in defence mechanisms against this reactive metabolite.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

"Fog Fever" or Acute Bovine Pulmonary Emphysema is probably the most important respiratory disease affecting grazing adult cattle in the United Kingdom. Until recently, the aetiology of the disease remained unclear and no suitable experimental model for the disease was available. However within the last ten years it has been established that certain indolic compounds when administered to cattle produce a disease syndrome similar, if not identical, to that produced by "Fog Fever" in the field (Carlson and Dickinson, 1978; Dickinson and Carlson, 1978). The aim of this work is to verify the validity of this experimental model and to investigate the mechanisms by which these indolic compounds bring about their pulmonary-toxic effect.

The "fog fever-like syndrome" can be experimentally induced by oral administration of the amino acid, L-tryptophan, which becomes converted in the rumen to 3-methylindole (skatole) which results in toxicity in the lung. In this work, the epidemiological, clinical and pathological features of the naturally-occurring disease were compared with experimentally-induced disease.

The hypothesis that excessive ingestion of L-tryptophan in grass is the cause of fog fever has been challenged because of the failure to identify a difference in concentration of L-tryptophan between pasture where the disease has occurred and normal pasture (Mackenzie, Ford and Scott, 1975; Selman, Breeze, Bogan, Wiseman and Pirie,

1977). Therefore an objective of this study was an investigation of the alternative hypothesis that altered conversion of L-tryptophan, rather than the excessive intake of the amino acid, to 3-methylindole was responsible. This was investigated by studying individual and daily variations in the ability of ruminal fluid to convert L-tryptophan to 3-methylindole (in vitro). Such quantative studies on the production of 3-methylindole by ruminal fluid samples from different animals might provide an explanation for the observed individual differences seen in response to oral dosing with L-tryptophan or, in the field, the individual differences seen in a group of animals after sudden change to better grazing.

The mechanism of production of 3-methylindole from L-tryptophan also requires investigation and also the efficacy of a number of compounds, including carbohydrate substances and antibiotics, for possible use as inhibitors of 3-methylindole production.

There are few substances which reproducibily cause specific pulmonary toxicity when administered by routes other than by inhalation. The mechanism for the specific organ toxicity of these substances has been poorly understood. However during the last few years the science of toxicology has seen major advances in the elucidation of the mechanisms by which foreign compounds induce organ-directed injury. It has become apparent that when a foreign compound induces specific organ damage this is often the result of formation of highly reactive metabolites. These reactive metabolites disrupt integrated biochemical pathways and/or alkylate cellular macromolecules in target organs (Gillette, 1974a, b). The reasons for the improvement in our understanding of these effects of highly

reactive metabolites are a result of recent techniques for the isolation of such transient metabolites and/or quantification of macromolecular-bound metabolite using radiolabelled precursors. In this present study, the possibility that 3-methylindole-induced toxicity is mediated through reactive metabolite(s) is investigated in detail. This study on the mechanism of action of 3-methylindole at the molecular level will assist not only in the elucidation of the aetiology of "fog fever" but it will contribute to our understanding in general, of organ-directed toxicity where the science of molecular toxicology is still in its infancy, and the number of well documented disease models is still small. It may also help to identify the possible effects in man of 3-methylindole which is known to occur in substantial amounts in the lower gut and in cigarette smoke (Wynder and Hoffman, 1967). The possible implication of 3-methylindole in the aetiology of pulmonary disease in man has already been suggested (Huang, Carlson, Bray and Bradley, 1977).

The effect of 3-methylindole and 3-methylindole analogues on isolated smooth muscles of the lung and on the release of mediators of anaphylaxis from chopped bovine lung preparations is also investigated. This work was conducted in view of suggestions that the pneumotoxic effect of 3-methylindole is due to pulmonary venoconstriction or the release of mediators of anaphylaxis (Breeze, 1973; Eyre, 1975).

CHAPTER 1

THE ROLE OF 3-METHYLINDOLE AND RELATED INDOLIC COMPOUNDS IN THE AETIOLOGY OF FOG FEVER

1.1 - FOG FEVER AS A SEPARATE DISEASE ENTITY

The disease fog fever has been known for a considerable time. The term fog fever is mentioned in an English veterinary textbook written by Topham in the eighteenth century, and was recognized by Knowlson in 1819 who described the disease as a respiratory distress disorder affecting cattle at pasture in the autumn. The word "fog" or "foggage" refers to the fourteenth century name of aftermath pasture, a grass that had been regrown after an earlier cut for hay, as the disease is known to affect cattle grazing on such a pasture (Breeze, Pirie, Selman and Wiseman, 1976).

The disease has been recognized in Europe for more than 100 years and in North America over the past 30 years under such names as Acute Bovine Pulmonary Emphysema, Pulmonary Adenomatosis, Bovine Asthma and Panting Disease (Maki, 1963). In Britain the name Fog Fever has been widely used. Selman, Wiseman, Pirie and Breeze (1974) and Pirie, Breeze, Selman and Wiseman (1974) emphasized that fog fever is a single disease entity. The disease has been defined as "a sudden onset respiratory distress syndrome with minimal coughing which occurs typically in hungry fat, beef-type cows, in the autumn, within two weeks of their being moved on to lush pasture" (Pirie, 1979).

The epidemiological, clinical and pathological features of the disease are discussed briefly below.

1.1.1 - Type of animal affected

It has been shown that fog fever, in Britain, is a disease problem of adult, beef-type cattle (Selman et al, 1974). In a

recent study, 138 grazing cattle of all ages which were claimed to be affected by fog fever - like conditions of sudden onset - were examined. Fog fever was confirmed in 43 adult cows, all of which were beef-type; 13 different pulmonary disorders accounted for the clinical signs in the other 45 adults (almost all of which were milking cows), none of which was affected by fog fever. The 50 immature animals, that is, those under two years of age at grass for the first or second time, which were examined in the same survey were all cases of parasitic bronchitis (Breeze, Pirie, Selman and Wiseman, 1975a). Another study was carried out over a four year period, during which 30 incidents of fog fever were investigated. Fog fever was not encountered in animals less than two years of age, and in most cases, affected cattle were more than four years old. In every instance affected cattle were females of the single suckling type and most were in extremely good condition. Some cases were in late gestation or had calves at foot. While most breeds and/or types encountered in the area in which the investigation was carried out were represented, the majority of affected animals were Herefords or Hereford crosses (Selman et al, 1974). These results and other work (Gibbons, 1962; Moulton, Cornelius and Osburn, 1963; Blake and Thomas, 1971) indicate that the disease is much more prevalent in cattle over two to three years of age. A possible explanation for the increased incidence in older cattle may be that repeated insults caused by this and other respiratory diseases result in a progressive decrease in pulmonary reserve. Consequently older animals may lose their capacity to resist the disease (Dickinson

and Carlson, 1978). The fact that fog fever appears to be more prevalent in beef herds may be explained in terms of grazing management since dairy cattle are usually maintained on a fairly uniform type of grazing and in general they do not experience the dramatic changes to which many beef herds are subjected during the later part of the grazing season (Selman et al, 1974). The incidence of fog fever is particularly high in Hereford or Hereford cross breed cattle (Barker, 1948; Cates, 1948; Selman et al, 1974). Reports of fog fever in dairy breeds (Roberts, 1927; Barker, 1948; Begg and Whiteford, 1948; Roberts, Benson and Jones, 1973) and in animals less than one year old (Barker, 1948) cannot be confirmed. The details provided do not exclude other disease possibilities. Selman et al (1974) pointed out that careful examination of cases claimed to be affected with fog fever in young or dairy breed cattle would reveal other pulmonary disorders.

1.1.2 - Seasonal occurrence of the disease and grazing history of affected herds

The most interesting feature of fog fever is its seasonal occurrence. Cases occur only in late summer and autumn (Cates, 1948; Begg and Whiteford, 1948; Roberts et al, 1973, Selman et al, 1974; Breeze et al, 1976).

Climatic changes claimed to be associated with incidents were the onset of warm, moist weather following a dry, cool period or the occurrence of cool nights following warm days (Roberts et al, 1973).

Seasonal incidence is apparently due to the practice of

transferring cattle from dry summer range to improved pastures in the late summer. This relationship to grazing management has been reported on many occasions by North American workers (Scofield, 1948; Goodman, 1956; Blood, 1962; O'Donoghue, 1960: Moulton, Harrold and Horning, 1961; Gibbons, 1962; Tucker and Maki, 1962; Moulton et al, 1963; Blake and Thomas, 1971). In Britain Selman et al (1974) emphasized the striking association between fog fever incidents and a recent abrupt change from poor to better grazing, the condition arising within two weeks of such a move. The authors point out that a change to better grazing could occur on a single field as a result of a sudden improvement in climatic conditions. Earlier British literature did not emphasize this relationship (Roberts, 1927; Barker, 1948; Leslie, 1949; Mackenzie, 1965; Roberts et al, 1973)

There is no clear relationship between grazing on a particular plant and the incidence of the disease. Most frequently the disease has been reported in cattle grazing lush aftermath pasture (Roberts, 1927; Barker, 1948; Leslie, 1949; Lambie, 1969; Selman et al, 1974). Cases of the disease have been reported in cattle grazing rape or stubble-turnips (Selman et al, 1974), on poor moor-edge grazings (Leslie, 1949), or even bare fields (Roberts et al, 1973).

1.1.3 - Possible relationship to soil management

Dickinson and Carlson (1978) claimed that marked differences in incidence occur between various ranges and water drainages and suggested that there may be a subtle difference in soil and water factors which affect plant growth and physiology. Preliminary work

indicated that soil samples from areas with outbreaks were generally lower in phosphorus, potassium and zinc content than those taken from "non-trouble" areas (Dickinson and Carlson, 1978). Selman et al (1974) emphasized that more than 80% of the fields on which fog fever arose had received at least one application of artificial fertilizer during the previous year, although it was mentioned that the amounts of nitrogen applied were not excessive by British national standards. Roberts et al (1973) reported that more than 50% of fog fever incidents arose on non-fertilized pastures. However, this cannot be confirmed as this report was based on data obtained largely by a retrospective questionnaire survey.

Selman et al (1974) suggested that the way in which the lush pasture was produced, that is by irrigation, fertilization, re-seeding, resting or even sudden improvement in climatic conditions was less important than the change itself.

1.1.4 Morbidity and mortality

The incidence of the disease in affected herds is extremely variable. Morbidity rates vary from a single animal to 100%, with marked variation in the severity of the disease between different affected animals (Tucker and Maki, 1962; Roberts et al, 1973; Selman et al, 1974). Mortality has been reported to vary from zero to 20%. Deaths usually occur within 72 hours after appearance of symptoms (Tucker and Maki, 1962; Breeze et al, 1976). Full rumen, pre-existing pulmonary lesions and physical exertion would be expected to worsen the respiratory embarrassment leading to death. Otherwise spontaneous recovery would occur (Tucker and

Maki, 1962; Dickinson, 1970; Selman et al, 1974; Dickinson and Carlson, 1978).

1.1.5 - Antemortem signs

Ante-mortem signs of fog fever are fairly characteristic. Signs usually become apparent within a few days after moving hungry, adult beef-type cattle from poor to better quality pastures. Severe respiratory distress serves to draw attention, although the level of supervision of grazing animals would often be insufficient to detect earlier or less severe signs. Two clinical forms of fog fever have been distinguished in Britain. The less severe form of the disease is characterized by slight tachypnoea and hyperpnoea. In the most severe form, the clinical signs include severe dyspnoea with a loud expiratory grunt, frothing from the mouth, mouth breathing and tachypnoea. Subcutaneous oedema may be observed in some animals. Coughing is not a dominant feature among affected individuals nor among other adults within an affected group (Selman et al, 1974).

1.1.6 - Pathology of fog fever

Pirie et al (1974) examined the lungs of animals which were confirmed cases of fog fever. Lungs of fatal cases of the disease were macroscopically and microscopically different from those of animals which were severely ill but appeared to be recovering up to the time when they were slaughtered in order to study the development of pulmonary lesions. In fatal cases of the disease predominant features were severe interstitial emphysema and pulmonary oedema. Additional findings included the presence of abundant frothy fluid

which filled the trachea, lobar and segmental bronchi, petechial and ecchymotic haemorrhages on and below the epithelial lining of larynx, trachea and bronchi. Congestion and oedema of the lamina propria of the conducting airways with accumulation of plasma cells and lymphocytes in this site. Hyaline membranes and early signs of alveolar epithelial hyperplasia were also observed. Non-fatal cases were further divided into two sub-groups: non-fatal cases with continuing marked clinical signs and mildly affected animals in which acute clinical signs had abated. The lungs of non-fatal cases of the disease with continued marked clinical signs were very different from those of fatal cases. Extensive alveolar epithelial hyperplasia and hyaline material were observed in these animals, but pulmonary oedema was largely absent. Focal lesions of mild pulmonary oedema and alveolar epithelial hyperplasia were seen in the lungs of mildly affected animals. These lesions were restricted in extent and degree of severity and were found to involve some segments in some lobes. It should be emphasized that Pirie et al (1974) did not regard the above-mentioned lesions of fog fever as pathognomonic, but certainly they were obligatory for diagnosis.

Further research was directed towards identifying the histogenesis of the alveolar epithelial hyperplasia. Based on the ultrastructural similarity between the hyperplastic cells and the type 2 pneumocyte (granular pneumocyte), it has been proposed that the alveolar epithelial hyperplasia, associated with fog fever, is due to proliferation of type 2 pneumocytes (Breeze, Pirie, Selman and Wiseman, 1975b).

Earlier reports of the disease either failed to record the pulmonary lesions in animals which were claimed to be cases of fog fever (Roberts, 1927; Barker, 1948; Leslie, 1949; Roberts et al, 1973) or used the term "fog fever" in a wider sense to include other forms of respiratory distress (Michel, 1954; Blood, 1962; Mackenzie, 1966).

Thus "fog fever" is a single disease entity. The term is reserved for a sudden onset respiratory syndrome with minimal coughing in adult beef-type cattle in the autumn soon after introduction to new pasture. The characteristic pulmonary lesions are any combination of pulmonary congestion, oedema, hyaline membranes, interstitial emphysema and alveolar epithelial hyperplasia (Selman, Wiseman, Pirie and Breeze, 1973; Pirie et al, 1974; Selman et al, 1974).

Selman et al (1973), Breeze et al (1975a) and Breeze et al (1976) pointed out that several authors used the term "fog fever" in a wider sense to include all forms of respiratory distress arising in all ages of cattle either indoors or outdoors. Confusion about the disease came from its obscure aetiology and also from the fact that acute respiratory distress is a feature of fog fever. It has been shown that a widely differing variety of conditions, of diverse aetiology, such as bovine farmer's lung, parasitic bronchitis, necrotizing pneumonia and carcinoma of the lung will all cause clinical signs of respiratory distress. Breeze et al (1975a) suggested that a new term Acute Respiratory Distress Syndrome might be useful for any sudden onset respiratory conditions with dyspnoea that is the result of any combination of the following pulmonary

lesions: congestion and oedema, hyaline membranes, alveolar epithelial hyperplasia, and interstitial emphysema. One specific form of acute respiratory distress syndrome would be fog fever, and it is the most important condition affecting adult cattle grazing (Pirie, 1977).

Several review articles on respiratory conditions of cattle are available (Breeze, 1973; Breeze et al, 1976; Pirie, 1977; Breeze, Selman, Pirie and Wiseman, 1978). Various theories on the aetiology of fog fever have recently been discussed (Selman, Wiseman, Breeze and Pirie, 1976).

1.2 - EXPERIMENTAL INDUCTION OF A RESPIRATORY DISTRESS SYNDROME SIMILAR TO FOG FEVER

1.2.1 - Experimental induction of a respiratory distress syndrome similar to fog fever by tryptophan and related indolic compounds

While the disease fog fever was characterized as a separate epidemiological, clinical and pathological entity, its aetiology remained unknown.

In 1965, an observation was made which led to productive experimentation in the study of the aetiology of fog fever. This unexpected event came about as a result of studies being performed on the activities of hepatic tryptophan pyrrolase enzyme in cattle and sheep. Following oral administration of large doses of the amino acid D,L-tryptophan, some of the cattle developed acute respiratory distress symptoms and post-mortem lesions were strikingly similar to those observed in the naturally occurring disease associated with the grazing of cattle on certain forage corps, sudden change of ration, or the feeding of mouldy feeds (Johnson and Dyer, 1966). These observations were confirmed by Dickinson, Spencer and Gorham (1967) who made two trials to induce the disease using eight mature cows of various ages and breeds. In both trials the cattle were maintained on a low plane of nutrition for a minimum of 30 days and then given a single oral dose of D,L-tryptophan (0.5-0.6 g/kg body weight). As early as 24 hours after treatment some of the treated animals developed symptoms of respiratory distress. This respiratory

distress was progressive in five animals, but slight in the other remaining three animals, and reached peak severity from four to seven days following treatment. Pulmonary lesions were found to be oedema, hyaline membranes, emphysema, proliferation of alveolar epithelial cells and infiltration of the lungs by neutrophils and eosinophils. Pathological changes were generally proportional to the clinical severity of the respiratory distress shown by the animal at the time of death. It was shown later that parenteral administration of tryptophan does not cause the pulmonary disease indicating that ruminal fermentation of tryptophan is required. Oral administration of D,L-tryptophan (0.7 g/kg) to cows caused interstitial emphysema 36 to 96 hours after treatment. The concentration of tryptophan in plasma was increased after tryptophan was administered but was apparently not directly responsible for development of the syndrome. Similar levels of tryptophan in plasma were produced in cattle by intraperitoneal injection and intravenous infusion, but these cattle did not develop interstitial pulmonary emphysema (Carlson, Dyer and Johnson, 1968). Oral administration of D-tryptophan (0.4 g/kg) did not cause the disease; only the L-isomer (0.35 g/kg) was effective in producing pulmonary disease (Carlson, Yokoyama and Dickinson, 1972) and then only by the oral route (Carlson et al, 1968). Thus it was proposed that a metabolite of L-tryptophan produced in the rumen is probably the active principle causing pneumotoxicity in tryptophan-treated cattle (Carlson et al, 1968). Thus subsequent research attempts were directed towards identifying a product of ruminal fermentation of L-tryptophan that is capable

of causing pulmonary oedema and emphysema in cattle.

After incubation *in vivo* and *in vitro* with rumen fluid, L-tryptophan was converted mainly to 3-methylindole with some indole and indoleacetic acid and this latter substance also formed 3-methylindole after further incubation (Carlson *et al*, 1972; Yokoyama and Carlson, 1974; Yokoyama, Carlson and Dickinson, 1975). Subsequently it was discovered that oral administration and intravenous infusion of 3-methylindole led to a pulmonary disease similar, but more acute in onset and course, to that produced by L-tryptophan administration (Carlson *et al*, 1972). Intravenous infusion of indoleacetic acid (0.07 g/kg) (Pirie, Breeze, Selman and Wiseman, 1976) and intraruminal administration of indole (0.4 g/kg) (Yokoyama *et al*, 1975) tryptamine and 5-hydroxytryptamine (Jarvie, Breeze, Selman and Wiseman, 1977) did not cause the disease.

Yokoyama *et al* (1975) measured plasma concentrations of 3-methylindole associated with tryptophan induced pulmonary disease. Five Hereford cows were given an intraruminal dose of L-tryptophan (0.35 g/kg). Three of the cows developed clinical signs of acute respiratory distress and showed severe pulmonary lesions when post-mortem examination was performed after 96 hours. Another cow developed moderate clinical signs and pulmonary lesions, and the remaining cow had few clinical signs and pulmonary lesions. 3-Methylindole was present in ruminal fluid and plasma within six hours after administration of tryptophan, and the concentrations increased to 3 and 9 µg/ml within 12 to 24 hours. Severity of pulmonary lesions was related to the maximal concentration and duration of 3-methylindole in the plasma. Post-mortem examination

showed gross pathological changes in the lungs characterized by diffuse pulmonary oedema and interstitial emphysema; and the lungs were dark red, firm and heavier than normal. The predominant microscopic lesions included pulmonary oedema, interstitial pulmonary emphysema and alveolar epithelial hyperplasia. This experiment clearly demonstrated that substantial amounts of 3-methylindole are produced in the rumen of cows given tryptophan, and concentrations comparable with those after oral 3-methylindole treatment can be detected in plasma. It was stated that the pulmonary lesions in cows given tryptophan or 3-methylindole are qualitatively similar and the severity is related to the concentration of 3-methylindole in the plasma.

Carlson, Dickinson, Yokoyama and Bradley (1975) investigated the effect of 3-methylindole in cattle. In three heifers given an intraruminal dose of 0.2 g/kg of 3-methylindole, clinical signs of respiratory disease appeared between 6 and 12 hours after dosing and death from pulmonary oedema and emphysera occurred at 72 hours. The mean plasma concentration reached a maximum (18.5 µg/ml) at three hours. In two cattle given an intraruminal dose of 0.1 g/kg of 3-methylindole signs of acute respiratory distress developed, but the animals did not die. The mean plasma concentration of 3-methylindole became maximal (16.8 µg/ml) at three hours. In three cattle given 0.06 g of 3-methylindole/kg by infusion into the jugular vein clinical signs appeared in all cows and one cow died. Post-mortem examination of these animals showed pulmonary oedema, interstitial emphysema and proliferation of the alveolar epithelial cells.

Pirie et al (1976) investigated the ability of 3-methylindole and indoleacetic acid to act as a direct proliferative stimulus on type 2 pneumocytes. Twelve one-year-old beef calves were divided into three equal groups. Two groups were given a single intravenous dose (0.07 g/kg) of indoleacetic acid or 3-methylindole over a twelve hour period and one group acted as a control group. Alveolar epithelial hyperplasia of type 2 pneumocytes was found in the lungs of all the calves in the group treated with 3-methylindole. Additional findings were interstitial emphysema, pulmonary oedema, focal hyaline membrane formation, pulmonary congestion, pulmonary eosinophilia, proliferation of terminal bronchial epithelial cells and numerous globule leucocytes in the epithelium of the trachea and bronchi. Alveolar epithelial hyperplasia, interstitial emphysema and pulmonary oedema were not observed in the lungs of the calves of other groups. Thus it was concluded that 3-methylindole, but not indoleacetic acid, can produce a proliferative alveolitis within 96 hours in the bovine lung. Eyre (1972) dosed ten cattle with L-tryptophan (0.5 or 1 g/kg) or a mixture of D,L-tryptophan (0.5-1 g/kg). Six out of ten animals treated with either regime showed varying degrees of respiratory distress. Surprisingly the author found that the most severe clinical reactions were in four of the youngest animals in the group (approximately six months old). All those animals of a year or older developed very mild transient signs or none at all. Animals receiving the highest dose of D,L-tryptophan (1 g/kg) showed no observable clinical signs. An elevation of 5-hydroxytryptamine concentration was demonstrated in plasma of affected and some of the normal animals. Based on these results, it was suggested that elevation in plasma

5-hydroxytryptamine concentration accompanying the excess dietary intake of tryptophan may be an aetiological factor in the development of acute bovine pulmonary emphysema of dietary origin. Results of this study cannot be interpreted with confidence. No histopathological examination was carried out. The possibility that the respiratory distress, displayed by these animals, was due to other causes unrelated to tryptophan cannot be excluded. The technique of spectrofluorimetric assay of 5-hydroxytryptamine of Anden and Magnusson (1967) used by the author is very delicate and its specificity in the case of L-tryptophan-dosed animals is questionable. The method is based on column chromatographic purification of 5-hydroxytryptamine followed by its spectrofluorimetric determination. Many indolic compounds including tryptophan and tryptophan metabolites have the same spectrofluorimetric characteristics of 5-hydroxytryptamine (Udenfriend, 1962; Anden and Magnusson, 1967). The column chromatographic procedure does not eliminate all these compounds (Anden and Magnusson, 1967). It is noteworthy that the author reported an increase in plasma 5-hydroxytryptamine not only in cattle with clinical signs of respiratory distress but also in apparently normal animals after tryptophan administration.

1.2.2 - Variations in the response of cattle to oral administration of tryptophan

There is a considerable individual and breed variation in the response of cattle to oral administration of L-tryptophan (Dickinson et al, 1967; Dickinson, 1970; Dickinson and Piper, 1971; Yokoyama et al, 1975). The clinical severity of the respiratory response is

variable, some individuals show a very severe response which often leads to death. Others have a mild to moderate transitory response and then apparently recover, whereas still others have little or no reaction following oral administration of L-tryptophan (Dickinson and Piper, 1971). Reproducibility of the experimental disease in cattle was apparently influenced by breed susceptibility, dosage levels of inducing agents, age and general conditions of the animals, extent of pre-existing lesions in the lungs and possibly other factors (Dickinson, 1970).

Monlux, Cutlip and Estes (1970) attempted to demonstrate differences in breed susceptibility to the tryptophan-induced lung damage. Twenty animals representing five breeds (Hereford, Jersey, Holstein, Angus and Shorthorn) of cattle were given oral doses of D,L-tryptophan (0.6 g/kg). Only Hereford (three out of four Herefords) and Jersey (two out of four Jerseys) developed the typical lesions of the disease, indicating that breed differences in the production of pulmonary disease in cattle do exist following the oral administration of tryptophan, with the Hereford breed being the most susceptible. However, it is noteworthy that the tryptophan-induced pulmonary disease has been reported in Guernsey, Shorthorn and Angus breeds (Dickinson et al, 1967) at the same dosage level used by Monlux et al (1970).

1.2.3 - Pasture levels of tryptophan associated with outbreaks of fog fever

It has been attempted to identify a difference in tryptophan concentration between pasture causing fog fever and normal pastures,

and to compare the tryptophan content of this pasture with the experimentally toxic dose of tryptophan (Mackenzie, Ford and Scott, 1975; Selman, Breeze, Bogan, Wiseman and Pirie, 1977). The amount of tryptophan consumed in the forage during naturally-occurring fog fever is not known. The disease usually arises in hungry cattle after being introduced to highly digestible lush pasture which they have not encountered for some time. This would result in a higher than normal feed intake during the first few days after such a pasture change. During this time changes in ruminal flora may be occurring to accommodate the increased amounts and the different type of forage (Carlson and Dickinson, 1978).

In a study on pasture content of L-tryptophan, associated with outbreaks of fog fever, there was no significant difference in tryptophan content between normal herbage from normal pastures and from pastures in which outbreaks of fog fever occurred. The concentration found was about 4 mg tryptophan per gram dry matter (Mackenzie et al, 1975).

It was concluded on theoretical grounds, based on comparison of the tryptophan content of grass, with the quantity of tryptophan required as a single dose to produce the experimental "fog fever-like" condition that ingestion of such grass would not provide sufficient tryptophan at a single feed for a direct toxic response. The minimal toxic amount of L-tryptophan would require to be ingested over a period of several days (Mackenzie et al, 1975; Selman et al, 1977).

1.2.4 - Experimental induction of fog fever by a sudden change of pasture

Selman et al (1977) conducted a trial to induce fog fever by a pasture change. Eleven adult beef-type cows were introduced to pasture free from Dictoyocaulus viviparus infection. All animals developed some evidence of respiratory disturbance. This became obvious between three and eleven days after the pasture change and lasted between one and five days. 3-Methylindole was present in the ruminal fluid of each animal in amounts ranging from 3.5-9.5 µg/ml but it was not detectable in plasma samples. Post-mortem examination showed pulmonary oedema, interstitial emphysema, alveolar epithelial hyperplasia and hyperplasia of the terminal bronchiolar epithelium (Selman et al, 1977).

Terry, Bradley, Hammond, Cummins, Carlson and Dickinson (1976) demonstrated that 3-methylindole can be produced by ruminal bacteria from cattle under field conditions and that 3-methylindole is present in blood plasma and ruminal fluid of cattle which developed acute bovine pulmonary emphysema under field conditions. Nineteen mature beef cows were moved from dry autumn range to lush green pasture to induce the disease. After four days, one cow developed severe and six cows developed moderate signs of fog fever. These signs regressed by the tenth day in the six cows with moderate signs. The cow with the severe signs was slaughtered on the fifth day and had the gross and microscopic lesions characteristic of the disease. 3-Methylindole was present in 84% of plasma samples (0.1 µg/ml-0.37 µg/ml) that had been collected over a one week period after pasture change. Ruminal

fluid 3-methylindole concentrations (3.1 µg/ml) in the cow with severe signs were ten times higher than plasma levels. Cultures of ruminal fluid from these cows converted indoleacetic acid and L-tryptophan to 3-methylindole.

1.2.5 - Inhibition of production of 3-methylindole, and the resultant respiratory distress, by antibiotics after oral administration of L-tryptophan or sudden change of pasture

Recently it has been shown that the antimicrobial agents, chlortetracycline and monensin, reduce 3-methylindole production and prevent the onset of respiratory distress after oral administration of L-tryptophan (Hammond, Carlson and Breeze, 1978; Hammond, Bray, Cummins, Carlson and Bradley, 1978). Monensin reduces ruminal 3-methylindole production in cows given access to lush pasture (Carlson, Hammond, Breeze, Potchoiba and Nocerini, 1981).

1.2.6 - Similarities between fog fever and the L-tryptophan-induced pulmonary disease

Oral administration of L-tryptophan and related indolic compounds produces a disease similar to "fog fever" in cattle. Thus an experimental model, which would induce the disease with a reasonable reproducibility, has been made available. It would be anticipated that exploitation of this model would allow a more rational approach to the control, prevention and treatment of the natural disease. However, a critical question is whether the experimental procedure produces a disease the same as that produced under natural conditions. The following similarities indicate that the experimentally-induced and the natural disease are identical and are the same disease.

A - Relationship to dietary factors

The experimentally-induced and the natural disease have a definite relationship to diet. In most outbreaks investigated, cattle at risk were suddenly able to consume unlimited amounts of very lush grass of a quality which they had not encountered for at least several months after abrupt change from poor to better grazing (Scofield, 1948; Blood, 1962; O'Donoghue, 1960; Moulton et al, 1961; Gibbons, 1962; Tucker and Maki, 1962; Moulton et al, 1963; Blake and Thomas, 1971; Selman et al, 1974). The experimental disease is produced by an overload of natural food constituents. L-Tryptophan is present in grass inducing the disease in substantial amounts (Mackenzie et al, 1975; Selman et al, 1977). Indoleacetic acid has been regarded as a plant growth-promoting substance or "plant hormone" and it is present in rapidly growing plants (Meister, 1965).

It was calculated that animals would ingest sufficiently toxic amounts of L-tryptophan over a period of several days. These calculations are based on comparison of the grass content of L-tryptophan with the experimentally toxic dose (Mackenzie et al, 1975; Selman et al, 1977). It should be emphasized that the minimal toxic dose of L-tryptophan is as yet unknown. A fair assessment of the role of L-tryptophan in the aetiology of fog fever would have to be based on comparison of the grass content of L-tryptophan with the minimum toxic dose of L-tryptophan.

The importance of previous diet has been emphasized for both the experimentally-induced and the natural disease. For

successful induction of fog fever animals have to be maintained on a low plane of nutrition for several weeks before dosing with L-tryptophan or introduction to lush pasture.

B - Similarity in epidemiological, clinical and pathological features

1. Onset: A time lapse of between 1 and 4 days occurs between dosing with tryptophan and the onset of clinical signs (Dickinson et al, 1967; Carlson et al, 1968). Fog fever incidents arise within two weeks after moving cattle from dry summer range to improved pastures in the late summer, with the first incidents of the disease occurring as early as 24 hours after such a move (Selman et al, 1974).

2. Ante-mortem signs: Ante-mortem signs of both the L-tryptophan-induced and the natural disease are similar. These are manifestations of acute respiratory distress with minimal coughing. In both the natural and the L-tryptophan-induced disease, the signs are extremely variable and may range from minimal distress to dyspnoea and death within 24 hours (Tucker and Maki, 1962; Dickinson et al, 1967; Dickinson, 1970; Dickinson and Piper, 1971; Yokoyama et al, 1975; Breeze et al, 1976).

3. Type of animal: The incidence of fog fever and the susceptibility to the pneumotoxic effect of orally administered tryptophan is particularly high in Hereford and Hereford-cross breed cattle (Barker, 1948; Cates, 1948; Monlux et al, 1970; Dickinson and Piper, 1971; Selman et al, 1974). The natural disease is much more prevalent in cattle over two to four years of age (Gibbons, 1962; Moulton et al, 1963; Blake and Thomas, 1971; Selman et al, 1974). Immature cattle are less susceptible to the effects of orally administered

tryptophan (Dickinson and Piper, 1971).

4. Pathological changes: In both the natural and the L-tryptophan-induced disease pathological changes are confined to the lung and are strikingly similar. Main findings include interstitial emphysema, pulmonary oedema, congestion, hyaline membranes and alveolar epithelial hyperplasia due to proliferation of type 2 pneumocytes (Johnson and Dyer, 1966; Dickinson et al, 1967; Pirie et al, 1974; Breeze et al, 1975b).

C - Relationship to 3-methylindole

It has been established that L-tryptophan has no direct pulmonary toxic effect; its ruminal metabolite, 3-methylindole, is responsible for the pneumotoxic effect of orally administered L-tryptophan. Production of abnormal amounts of 3-methylindole is associated with respiratory distress after abrupt change from poor to better grazing or oral administration of L-tryptophan. In both cases the severity of symptoms and post-mortem lesions are related to plasma 3-methylindole concentration (Yokoyama et al, 1975; Terry et al, 1976).

CHAPTER 2

STUDIES ON THE METABOLISM OF L-TRYPTOPHAN TO 3-METHYLINDOLE IN CATTLE

2.1 - INTRODUCTION

2.1.1 - The metabolism of L-tryptophan by ruminal microorganisms

A - Incorporation into microbial proteins

Tryptophan becomes incorporated into ruminal microorganisms probably in the form of microbial proteins. This incorporation does not occur to a great extent. Less than 4% of radioactivity was incorporated into ruminal microorganisms after incubation of ruminal fluid for 24 hours with labelled L-tryptophan (Candlish, Devlin and La Croix, 1970; Yokoyama and Carlson, 1974).

B - Conversion to other indolic compounds

Lewis and Emery (1962a) divided amino acids into three groups with regard to their relative rates of deamination by rumen microorganisms. Tryptophan was in the group in which deamination was less pronounced.

The major end products of D,L-tryptophan metabolism in rumen fluid found in an early study (Lewis and Emery, 1962b, c) were indole and 3-methylindole. Using radiolabelled tryptophan, it has recently been shown that 3-methylindole and to a lesser extent indole and indoleacetic acid are the major indolic metabolites of L-tryptophan fermentation by ruminal microorganisms in vitro. Indoleacetic acid is metabolized further to 3-methylindole, but not to indole. Based on these findings Yokoyama and Carlson (1974) proposed that the major route by which 3-methylindole is formed from L-tryptophan is by a two step process involving the initial formation of indoleacetic acid, followed by subsequent decarboxylation

of indoleacetic acid to 3-methylindole. D-tryptophan was not converted to any of these metabolites (Yokoyama and Carlson, 1974). Scott, Ward and Ward (1963) showed that incubation of D,L-tryptophan in the artificial rumen for one hour resulted in about 3% conversion to indoleacetic acid. Schatzmann and Gerber (1972) reported on the formation of tryptamine from L-tryptophan by viable ruminal fluid. The yield was about 0.001% conversion after four hours incubation.

C - Microorganisms involved in the production of 3-methylindole

Inhibition of conversion of L-tryptophan to 3-methylindole by antibiotics strongly suggests that this conversion is due to bacterial activity. The role of ruminal protozoa appears to be far less significant (Yokoyama and Carlson, 1974). A bacterium was isolated from bovine ruminal fluid which is capable of decarboxylating indoleacetic acid to 3-methylindole, but it does not metabolize tryptophan to 3-methylindole. This organism is a gram positive, non-motile, non-spore forming bacillus. It is a strict anaerobe. The organism has been assigned to the genus lactobacillus (Yokoyama, Carlson and Holdeman, 1977).

D - Synthesis of tryptophan by ruminal microorganisms

It has been shown that rumen microorganisms can synthesize tryptophan from non-protein nitrogen (ammonium phosphate). Nicotinic acid is an essential requirement for this synthesis (Piana and Piva, 1969).

Tryptophan synthesis from indoleacetic acid by anaerobic bacteria from the rumen was demonstrated by Allison and Robinson (1967) who reported that radioactivity from radiolabelled indoleacetic acid became incorporated into microbial protein in the form of

tryptophan.

2.1.2 - Absorption of tryptophan after oral administration in cattle

Ruminal fluid concentration of D,L-tryptophan was not markedly reduced until 8-10 hours after oral administration of D,L-tryptophan in cattle (Lewis and Emery, 1962c). These findings indicate that tryptophan is not rapidly absorbed following oral administration in cattle.

Plasma tryptophan concentration reached a maximum at 4-8 hours, with some variation in the concentration of tryptophan in plasma and in the time of maximum concentration after oral administration of tryptophan (Carlson et al, 1968). Studies using radiolabelled tryptophan have shown that the first detectable radioactivity appeared in the urine of cows about 2-3 hours after dosing with labelled tryptophan. More than 50% of the total radioactivity was excreted by twelve hours after dosing. Most of the radioactivity had been excreted by 24 hours after dosing. Radioactivity was excreted in the urine until 42-60 hours after dosing (Yang and Carlson, 1972).

In sheep, when tryptophan was injected into the rumen, 25-70% of the free tryptophan in the rumen was absorbed into portal blood within three hours. Tryptophan appears to be absorbed from the rumen and reticulum at significant rates and the only apparent limitation to the absorption is the concentration of tryptophan in the rumen fluid (Candlish, Stranger, Devlin and La Croix, 1970).

2.1.3 - Post-absorption metabolism of tryptophan in cattle

In all mammalian species investigated a major pathway of tryptophan metabolism is through kynurenine. There are two enzymes

known to be involved in the conversion of tryptophan to kynurenine. Tryptophan pyrrolase mediates the cleavage of the pyrrole moiety of tryptophan. The product of this reaction, N-formylkynurenine, is converted to kynurenine under the influence of kynurenine formamidase (formylase). Knox and Mehler (1951) found the latter enzyme to be in 600-fold excess in liver preparations and indicated that any change in kynurenine formation was therefore due to activity of the pyrrolase. It has been shown repeatedly that there is an increased conversion of tryptophan to kynurenine upon administration of tryptophan indicating an adaptive increase in tryptophan pyrrolase. The kynurenine pathway of tryptophan metabolism and tryptophan pyrrolase adaptation has been extensively studied in man and monogastric animals (Price, Brown and Yess, 1965; Meister, 1965; Brown and Price, 1956; Leklem, Woodford and Brown, 1969; Leklem, 1971; Leklem, Brown, Hankes and Schmeeler, 1971). Other supposedly minor pathways of tryptophan metabolism include hydroxylation of tryptophan to 5-hydroxytryptophan, then decarboxylation of 5-hydroxytryptophan to 5-hydroxytryptamine (Udenfriend, Titus, Weissbach and Peterson, 1956); and decarboxylation of tryptophan to tryptamine (Weissbach, King, Sjoerdsma and Udenfriend, 1959); tryptamine becomes converted to indoleacetaldehyde and then to indoleacetic acid (Johnson and Dyer, 1968).

Lacking evidence to the contrary, it has been assumed that the intermediate metabolism of tryptophan in the ruminant is similar to that in other organisms. Newer evidence from recent studies indicates that differences exist between ruminants (bovines and

ovines) and other animal species with regard to the metabolism of tryptophan. It has been shown that liver tryptophan pyrrolase activity is lower in ruminants (bovines and ovines) than in rats, and that large doses of prednisolone and tryptophan do not cause appreciable enzyme induction in these species within the same time period that is effective in rats (Carlson and Dyer, 1970). Cattle affected with pulmonary toxicity after oral doses of tryptophan were found to have a reduced liver tryptophan pyrrolase activity in the liver. This unexpected effect was attributed to the early toxic effect of orally administered tryptophan in cattle (Johnson and Dyer, 1966).

The results of Johnson and Dyer (1968) and Yang and Carlson (1972) cast doubts on the relative importance of the kynurenine pathway of tryptophan metabolism in the bovine. Several of the metabolites from the pathways presumed to be minor were excreted in as great or greater quantity than was kynurenine following intramuscular (Johnson and Dyer, 1968) and intraruminal (Yang and Carlson, 1972) administration of radiolabelled tryptophan.

Tryptophan, 5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, tryptamine, indoleacetic acid and kynurenine were excreted in urine following intramuscular administration of tryptophan. The pattern of excretion of tryptophan metabolites in tympanitic cattle was different from that in normal cattle (Johnson and Dyer, 1968). Yang and Carlson (1972) investigated the effect of high doses of L-tryptophan and different nutritional planes on the urinary excretion of tryptophan metabolites in order to

determine whether specific tryptophan metabolites are associated with the pneumotoxic effect of orally administered L-tryptophan. Urinary tryptophan metabolites were examined by ion exchange chromatography. In this experiment kynurenine, tryptophan, hydroxyindole derivatives, picolinic acid, acetyltryptophan, indole-3-acetic acid, 5-hydroxy-indole-3-acetic acid, oxindole derivatives and indican were identified in urine after intraruminal administration of radiolabelled tryptophan. Thus, products of tryptophan degradation as indole and oxindole derivatives were excreted in amounts greater than metabolites resulting from the kynurenine pathway of tryptophan metabolism following oral administration of tryptophan in cattle. A high dose of tryptophan resulted in an increased proportion of the total urinary metabolites being excreted as tryptophan, kynurenine, acetyltryptophan, 5-hydroxy-indole-3-acetic acid and indican in the urine compared with cattle receiving a low dose. Also indoleacetic acid and 5-hydroxyindole-3-acetic acid were more rapidly excreted in the cow given the high dose of tryptophan than in the cow given the small dose.

5-Hydroxyindole-3-acetic acid was absent in the urine of cattle which had been maintained on concentrate ration prior to tryptophan dosing. These cattle excreted more kynurenine and acetyl-tryptophan than cattle which had been maintained on hay prior to tryptophan dosing. These latter cattle excreted higher amounts of 5-hydroxyindole-3-acetic acid, hydroxyindole derivatives and two unidentified metabolites. Thus it was concluded that the plane of nutrition and high doses of tryptophan alter the urinary excretion pattern of tryptophan metabolites in cattle (Yang and Carlson, 1972).

Pamukcu, Brown and Price (1959) identified and quantitatively

measured the urinary excretion of α-aminohippuric acid, anthranilic acid, kynurenine, hydroxykynurenine, kynurenic acid and xanthurenic acid in healthy cattle and in cattle with urinary bladder tumours. Except for higher amounts of N-acetylkynurenine in the urine of cattle with tumours, the levels of most tryptophan metabolites in the urine were not different between normal and tumour-bearing cattle.

In conclusion, tryptophan metabolism in cattle is different from monogastric animals. Apart from differences arising from anatomical peculiarities of cattle, it has been shown that liver tryptophan pyrrolase activity is lower in ruminants than in monogastric animals (Carlson and Dyer, 1970). This enzyme is a key point regulating the traffic of tryptophan through various pathways (Knox, 1966). Unlike in monogastric animals, this key enzyme in ruminants does not undergo marked adaptive increase by administration of the appropriate substrate (Carlson and Dyer, 1970). Therefore the relative importance of the kynurenine pathway of tryptophan metabolism in cattle is less than that in monogastric animals (Johnson and Dyer, 1968; Yang and Carlson, 1972). Tympany, bladder tumours and ration affect the pattern of urinary excretion of tryptophan metabolites in cattle (Pamukcu et al, 1959; Johnson and Dyer, 1968; Yang and Carlson, 1972).

2.1.4 - The toxic effect of indole and 3-methylindole on ruminal microorganisms and on certain enteric bacterial species

Wood, Gunsalus and Umbreit (1947) showed that the metabolism of tryptophan to indole, by certain enteric bacteria is inhibited by indole. Tittsler and Sandholzer (1935) and Tittsler, Sandholzer

and Callahan (1935) found that small concentrations of indole or 3-methylindole possesses a definite antibacterial action on many of the enteric bacilli. Various species and even individual strains of the same species exhibited well defined differences in their tolerance for indole. No significant correlation between sensitivity to indole and the production of indole by bacterial growth was discovered. The potency of 3-methylindole was approximately twice that of indole. Species differences in susceptibility to 3-methylindole were less marked than those observed with indole. It was suggested that a combined action of both indole and 3-methylindole would control the character of enteric bacterial flora.

Both indole and 3-methylindole cause disruption and disintegration of rumen protozoa and, because of its higher lipid solubility, 3-methylindole is more potent than indole (Eadie and Oxford, 1954; Bailey and Howard, 1962; Bailey and Russel, 1965).

It has been suggested that the presence of indole and 3-methylindole in ruminal fluid may provide an explanation for the variation in density and composition of ciliate protozoa in the normal rumen which occur from time to time (Eadie and Oxford, 1954).

2.1.5. - Inducibility of the tryptophanase enzyme of certain enteric bacteria

Happold and Hoyle (1935) described the preparation of a non-viable suspension of Escherichia coli which would convert tryptophan into indole in the presence of oxygen. The enzyme involved was named tryptophanase. The development of tryptophanase by the organism is controlled in part by the concentration of free tryptophan in the

medium. The tryptophanase enzyme in this preparation appears to be different from that of ruminal microorganisms since the tryptophanase (Escherichia coli) cannot produce indole from indole pyruvic acid (Happold and Hoyle, 1935) while that from ruminal microorganisms can (Yokoyama and Carlson, 1974).

2.1.6 - The inhibitory effect of antibiotics and certain other compounds on the production of 3-methylindole by ruminal fluid

Yokoyama and Carlson (1974) investigated the effect of a number of antibiotics on 3-methylindole production in vitro. Incorporation of polymyxin B, chlorotetracycline and oxytetracycline strongly inhibited 3-methylindole production from L-tryptophan in ruminal fluid. Oleandomycin, penicillin G, kanamycin and neomycin were less effective, while streptomycin was not effective. Hammond, Carlson and Breeze (1978) evaluated the possible inhibitory effect of the polyether antibiotics monensin and lasalocid, and a variety of compounds known to have anti-metabolite properties on the production of 3-methylindole in vitro. A concentration of 25 µg/ml of monensin, lasalocid, 4,4 dimethyldiphenyl idonium chloride, diphenylidonium chloride, 1,1,1-trichloro-2-hydroxy-4-pentanone, dichlorolurea, amichloral, dithiooxamide reduced 3-methylindole formation in vitro.
In addition monensin was shown to reduce 3-methylindole formation in vivo and thereby to protect cattle against the L-tryptophan-induced acute respiratory distress. In their study Hammond, Carlson and Breeze (1978) divided eight Hereford cows into two groups (four cows each) and gave 0.35 g of L-tryptophan/kg of body weight to induce acute bovine pulmonary emphysema. One group was treated with

L-tryptophan alone (control) while each of four cows in the other group was given 100 mg of monensin in gelatin capsules twice daily starting one day before and ending four days after the tryptophan dose. Mean ruminal concentrations of 3-methylindole in control cows reached maximum concentrations (mean = 36.4 µg/ml) at 12 hours and persisted at concentrations above 15 µg/ml from 3 to 36 hours after the cows received tryptophan. Mean concentrations in monensin-treated cows never exceeded 5.0 µg/ml. Three of the four control cows were severely affected and had pulmonary lesions of tryptophan-induced pulmonary disease. The fourth control cow had mild clinical signs and only focal lesions. None of the monensin-treated animals had clinical signs or pulmonary lesions of tryptophan-induced pulmonary disease. Hammond, Bray, Cummins, Carlson and Bradley (1978) explored the possible use of chlortetracycline and molasses to prevent the L-tryptophan-induced bovine pulmonary emphysema. Three groups of cows (six animals each) were used. One group was given tryptophan (0.35 g/kg) alone, another group was given tryptophan (0.35 g/kg) and chlortetracycline (4 g 24 hours and 12 hours prior to tryptophan administration followed by 2.5 g every 12 hours for three days after tryptophan administration), and a third group was given tryptophan (0.35 g/kg) and molasses (0.7 kg every 12 hours for four days). Ruminal 3-methylindole concentration reached a maximum above 15 µg/ml between 18 and 24 hours for the L-tryptophan alone and the L-tryptophan-molasses-treated cows, but remained less than 1 µg/ml throughout the experiment for the L-tryptophan-chlortetracycline-treated cows. Three cows from the tryptophan alone treated group

developed acute respiratory distress and one of these died at five days after tryptophan administration. Two cows from the L-tryptophan-molasses-treated group developed clinical signs of acute respiratory distress and both died at five days. None of the cows in the L-tryptophan-chlortetracycline-treated group developed any signs of acute respiratory distress.

It was later shown that monensin can reduce 3-methylindole production in the ruminal fluid of cows given access to lush pasture which suggested that it may be effective in preventing naturally occurring fog fever (Carlson, Hammond, Breeze, Potchoiba and Nocerini, 1981). To investigate this possibility Carlson, Hammond, Breeze, Potchoiba and Nocerini (1981) divided thirty Hereford cows into two equal groups and introduced them to lush pasture after they had been maintained on poor quality hay for three weeks. The test group were given a daily dose of 200 mg monensin in a gelatin capsule and the control group were each given a placebo starting one day before introduction to the lush pasture and continued for eight days. Ruminal 3-methylindole concentrations were significantly lower in the monensin group at all times except at day 10 which was three days after the last monensin dose. One control cow developed severe clinical signs of fog fever (Carlson, Hammond, Breeze, Potchoiba and Nocerini, 1981). The effects of monensin pre-treatment on ruminal 3-methylindole production and induction of the L-tryptophan-induced pulmonary disease has recently been investigated. Results of recent experimental work (Hammond, Carlson and Breeze, 1981) indicate that for effective prevention of acute pulmonary emphysema, monensin administration must be continued after initial exposure to L-tryptophan. Oral administration of

monensin (200 mg per animal) for two weeks and ending two days before tryptophan treatment did not prevent the onset of acute respiratory distress after oral administration of L-tryptophan in cattle. It should be emphasized that antibiotics and idonium compounds are not restricted in their influence to the particular metabolic pathway leading to the formation of 3-methylindole from L-tryptophan, but are rather general. Inhibition of degradation of amino acids by ruminal microorganisms has been reported for potassium penicillin G (Lewis and Emery, 1962a) and idonium compounds (diphenylidonium chloride and 4;4-dimethyl-phenylidonium chloride) (Chalupa, Patterson, Chow and Parish, 1976). Also monensin has been reported to have some bacteriostatic effects and to reduce feed consumption when given to cattle (Chalupa, 1979; Raun, Colley, Rathmacher, Richardson and Potter, 1974). The improved performance of monensin-fed beef cattle is probably due to the effect of monensin on the metabolism of volatile fatty acids by ruminal microorganisms. Monensin increases the relative proportion of propionic acid compared to acetic acid and butyric acid production in the rumen of cattle (Perry, Beeson and Mohler, 1976).

2.1.7 - Possible modification of 3-methylindole production by carbohydrates

A recent study (Yokoyama and Carlson, 1974) has shown that addition of glucose to ruminal fluid caused a marked reduction in the formation of both 3-methylindole and indole from L-tryptophan. Earlier literature reported that several bacterial enzymes of diverse properties are affected when bacterial cells are grown in a glucose

containing medium, and that this effect is not specific to glucose, but shared by other fermentable carbohydrates.

Presence of glucose in the incubation medium caused inhibition of the tryptophanase activity of Escherichia coli (Happold and Hoyle, 1936). Evans, Handley and Happold (1942) claimed that the tryptophanase system is adaptive, and that the previous nutritional history of the cell during growth determines its activity. The authors found that incorporation of arabinose, lactose, glucose, fructose, mannitol and potassium d-gluconate in the incubation medium of Escherichia coli caused inhibition of indole production with a reduction in the pH. It was concluded that inhibition of the tryptophanase activity was not due to pH changes because acid production without marked inhibition of indole production was obtained with rhamnose, glucosamine hydrochloride, xylose, sorbitol, galactose, d-ribose and mannose. Bacteria grown on carbohydrates (glucose, lactose, xylose or maltose) displayed a markedly reduced tryptophanase activity. Tryptophanase activity of such cells could be restored by the addition of certain amino acid mixtures, but not by vitamin B, which would indicate that carbohydrate utilization resulted in prevention of tryptophanase formation directly or indirectly by causing a deficiency in an essential nitrogen source concerned in enzyme synthesis (Boyd and Lichstein, 1955). Fildes (1938) reported that the tryptophanase of Escherichia coli can be divided into a small constitutive portion and a large adaptive portion, and that the presence of glucose during growth inhibits the formation of the adaptive portion of the enzyme.

Kendal and Farmer (1912, 1913) showed that on growing cultures

of a number of different bacterial species the ammonia liberated from protein digests decreases or even disappears when carbohydrate is present. Kendall (1922) suggested that this effect was due to the protein sparing action of carbohydrate. Berman and Rettger (1918) showed that the excretion of protease by Proteus vulgaris is also inhibited by the presence of carbohydrate in the medium, but since the effect is less marked with an organism such as Bacillus subtilis which produces little acid from glucose, they suggested that the effect was due to production of acid from fermentation of the carbohydrate. The addition of buffer to the medium decreased the inhibitory effect of glucose and this supports the theory that the effect is one of pH. Raistrick and Clark (1921) pointed out that the growth of many bacterial species is much greater in the presence of glucose in the medium and suggested that the greater yield of cell nitrogen accounts for some of the missing ammonia nitrogen, but this factor was insufficient to explain the discrepancy in most cases.

Stephenson and Gale (1937) found that the addition of glucose to a washed suspension of Escherichia coli grown in the absence of glucose has no significant effect on activity of the glycine, alanine and glutamic acid deaminases, but if the suspension is grown in the presence of 2% glucose, the resulting suspension will have only 10-20% of the deaminase activities of those grown in its absence. The effect of glucose is therefore not on the course of the enzyme action after growth is complete but inhibits the formation of the deaminases during growth. These results were also obtained with dl-serine deaminase (Gale and Stephenson, 1938) and aspartase (Gale, 1938).

Epps and Gale (1942) reported that in the presence of glucose in the growth medium of Bacillus coli, the enzyme glucozymase undergoes adaptive increase. The enzymes hydrogenase, catalase, arginine, lysine and histidine decarboxylases are not affected, while the formation of the enzymes ornithin decarboxylase, alanine, glutamic acid and serine deaminases, aspartase, formic, succinic and alcohol dehydrogenases and tryptophanase are suppressed. The reduction of activity of these enzymes as a result of growth in glucose is not a permanent change in the enzyme constitution of the cell as the effect is removed immediately when growth takes place in the absence of fermentable carbohydrates. Neutralization of the fermentation acids during growth in glucose does not alter the degree of inhibition of deaminase formation produced by the glucose. Moreover the reduction in aspartic acid deaminase activity in cells harvested from a medium containing glucose has been shown not to be specific to this sugar, but rather is shared with other fermentable carbohydrates; the results obtained with citrate were particularly revealing for it exerted a definite inhibitory action on the aspartate deaminase activity in spite of the fact that there was an increase in pH during growth rather than a decrease as is noted with the other carbohydrates utilized by the organism. The nature of the medium (pH) had little effect on the deaminase activity of the cells or on the inhibitory action of the added carbohydrates (Boyd and Lichstein, 1953). Boyd and Lichstein (1951) showed that glucose inhibition of aspartic acid, threonine and serine deaminases is not primary on apoenzyme production since a large excess of preformed co-enzyme or co-enzyme precursors when added to the glucose-containing medium caused at least partial

restoration of the lost deaminase activity. These results are consistent with an interpretation that the presence of glucose during growth either prevents the formation of the co-enzyme of these deaminases or causes its destruction.

2.2 - EXPERIMENTAL

2.2.1 - Introduction

It has previously been shown that the L-tryptophan-induced pulmonary disease is similar to fog fever (see Chapter 1). However the theory that pasture L-tryptophan is the cause of naturally occurring fog fever has been frustrated by failure to identify sufficiently high amounts of soluble tryptophan, in grass, that would allow the animal to ingest at a single feed quantities of tryptophan comparable to the experimental toxic dose of the amino acid inducing the disease (Mackenzie et al, 1975; Selman et al, 1977). An accurate assessment for the aetiological role of L-tryptophan, in naturally occurring fog fever, would have to consider the follcwing:

1) The grass content of L-tryptophan compared with the minimal effective dose. The minimal effective dose which is not established is difficult to determine since the response of cattle to orally administered L-tryptophan is extremely variable (Dickinson et al, 1967; Dickinson, 1970; Dickinson and Piper, 1971; Yokoyama et al, 1975).

2) L-tryptophan has no direct pulmonary toxic effect (Carlson, Dyer and Johnson, 1968). 3-Methylindole, a ruminal metabolite of L-tryptophan, is responsible for the pulmonary toxic effect of orally administered L-tryptophan (Carlson et al, 1972; Yokoyama and Carlson, 1974; Yokoyama et al, 1975). Studying the kinetics of conversion of the non-toxic parent compound L-tryptophan to the toxic metabolite 3-methylindole would provide a better assessment of the role of

of L-tryptophan in the aetiology of fog fever and would help to explain the variable response of cattle to orally administered tryptophan or sudden change to better grazing.

In this investigation the conversion of L-tryptophan to 3-methylindole by ruminal fluid samples from different animals was measured.

3) It has been shown that 3-methylindole is toxic to ruminal protozoa and enteric bacilli (Tittsler, Sandholzer and Callahan, 1935; Eadie and Oxford, 1954; Bailey and Howard, 1962; Bailey and Russel, 1965). Using ^{14}C-labelled tryptophan as a substrate, the effect of 3-methylindole on the conversion of L-tryptophan to 3-methylindole was measured.

4) One of the possible methods of prevention of fog fever would be to inhibit the conversion of L-tryptophan to 3-methylindole. In this study the observations of Yokoyama and Carlson (1974) that some antibiotics and glucose inhibit ruminal production of 3-methylindole from L-tryptophan were extended to examine the percentage inhibition of conversion at different antibiotic concentrations and with other carbohydrates and carbohydrate related compounds.

5) Although L-tryptophan is well documented as producing a "fog fever-like" syndrome when administered orally to cattle by other workers, this had not been successfully achieved in the Glasgow Veterinary School or in any other British establishment. A trial was therefore mounted to examine whether L-tryptophan would induce such a syndrome under the conditions prevailing in the United Kingdom. This experiment was conducted in collaboration with the Department of

Veterinary Medicine.

2.2.2 - Materials

Indole, 3-methylindole, L-tryptophan, indoleacetic acid, penicillin G sodium, streptomycin sulphate, ampicillin, tetracycline hydrochloride, chloramphenicol, sulfaguanidine and sulfamethoxy-pyridazine were obtained from Sigma Chemical Co. Ltd., London. N-hexane, mannitol, inulin, starch, galactose, glucose, fructose, lactose, sucrose, glycerin, trisodium citrate, sodium acetate, sodium lactate, silica gel G type 60, cyclohexane, chloroform, diethylamine, P-dimethylaminobenzaldehyde, formaldyhyde and trichloroacetic acid were obtained from BDH Chemicals Ltd., Poole, Dorset, England. "Cremophor-EL" was a gift from Victor Blagden & Co., Croydon, Surrey, England. The liquid scintillation cocktail LSE93 was obtained from Koch-Light Ltd., Colnbrook, Buckinghamshire, England. Radiolabelled tryptophan was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England.

2.2.3 - Ruminal fluid

Ruminal fluid was obtained from three cows (4-6 years old, Hereford-cross breed, designated A, B and C) with permanent ruminal fistulae, and maintained on hay and commercial concentrate mixture. The cows were housed indoors and had been maintained on this diet for a period of six months prior to the experimental period. Samples of ruminal fluid were obtained at 10 a.m. Ruminal fluid was strained through four layers of cheese cloth and used immediately for the experimental work.

Boiled ruminal fluid was prepared by boiling ruminal fluid

for fifteen minutes (boiling water bath).

2.2.4 - Incubation conditions

Incubation of ruminal fluid was carried out in 25 ml capacity conical flasks. Each flask was fitted with a rubber stopper through which two needles (gauge No. 16) were inserted. Flasks were attached in groups of four (Fig. 2.1). All incubations were done under anaerobic conditions (carbon dioxide gassing for 30 minutes) in a shaking water bath at 37°C. Samples were taken by breaking the seal. Carbon dioxide regassing for 30 minutes was carried out after taking samples. 85% phosphoric acid was added to samples (one drop/ml), mixed and stored (-20°C) until determination of 3-methylindole which was made within three days.

2.2.5 - Gas liquid chromatographic determination of 3-methylindole in ruminal fluid and plasma

A sample (1 ml) of ruminal fluid or plasma was transferred to a 12 ml glass stoppered test tube, 10 ml of redistilled n-hexane were added and the extraction tube was rotated for five minutes (mechanical shaker). 5 ml from the upper hexane layer were transferred to a graduated centrifuge tube and evaporated to 0.5 ml under an atmosphere of nitrogen at 40°C. 5 µl were used for injection into the gas chromatograph. 3-Methylindole concentrations were quantified from known standards.

Gas-liquid chromatographic determination of 3-methylindole was made according to the procedure of Bradley and Carlson (1974) as described by Atkinson, Bogan, Breeze and Selman (1977) as follows:

a Pye 104 gas-liquid chromatograph equipped with a hydrogen flame ionization detector and a glass column (1 m x 5 mm) packed with 10% DC200 on Gas-Chrom Q (100-200 mesh) was used at a column temperature of 130°C and detector temperature of 250°C. The carrier gas was nitrogen at a flow rate of 60 ml/minute. A series of standard solutions of indole and 3-methylindole were prepared in redistilled n-hexane. A 5 µl aliquot was injected into the chromatograph. The standard curve was prepared using the height of the peaks.

Recoveries

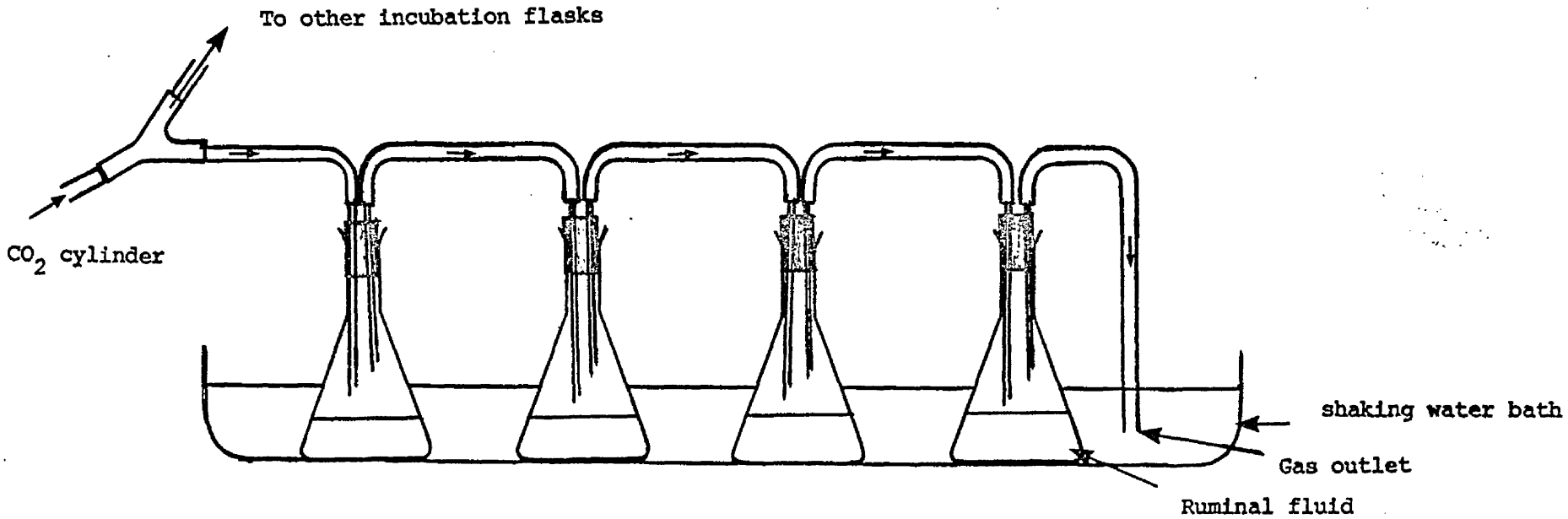
Four different concentrations of 3-methylindole in ruminal fluid or plasma (5, 10, 20 and 40 µg/ml) were prepared by dilution from a stock solution of 3-methylindole containing 100 µg/ml in 1% Cremophor-EL in distilled water. Duplicate 1 ml samples of these solutions were treated and 3-methylindole determinations were conducted as described above and recovery of 3-methylindole was calculated.

Results

A typical gas chromatogram for a standard solution containing 3-methylindole and indole in hexane is shown in Fig. 2.2. The resolution of indole and 3-methylindole was excellent. The recorder response (peak heights) was linear for various concentrations of 3-methylindole (Fig. 2.2). As shown in Fig. 2.3, none of the constituents of ruminal fluid or plasma interfered with the assay.

Recovery of 3-methylindole from ruminal fluid was 94 ± 1% (mean ± S.E.M.). Recoveries of 3-methylindole from ruminal fluid and plasma are shown in Tables 2.1 and 2.2 respectively.

Fig. 2.1 An assembly for anaerobic incubation of ruminal fluid



Assembly for anaerobic incubation of ruminal fluid at 37°C

Fig. 2.2 A gas-chromatogram of a standard solution of a mixture of 3-methylindole (3MI) and indole (I) in n-hexane and a series of standard solutions of 3-methylindole in n-hexane

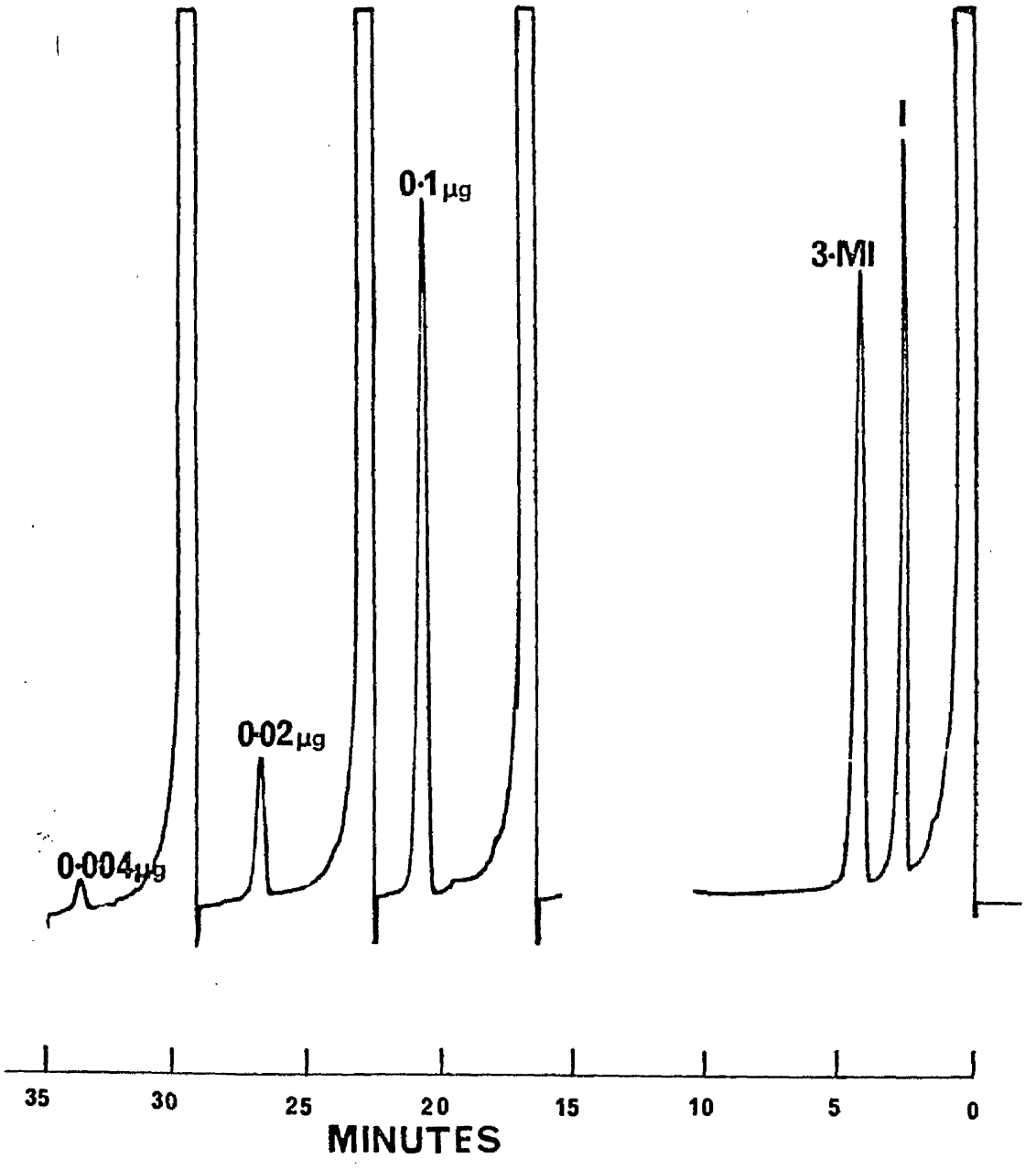

0·004µg
0·02µg
0·1µg
3·MI
I
35
30
25
20
15
10
5
0
MINUTES

Fig. 2.3 A gas-chromatogram of 3-methylindole (3MI) recovered from rumen fluid and plasma

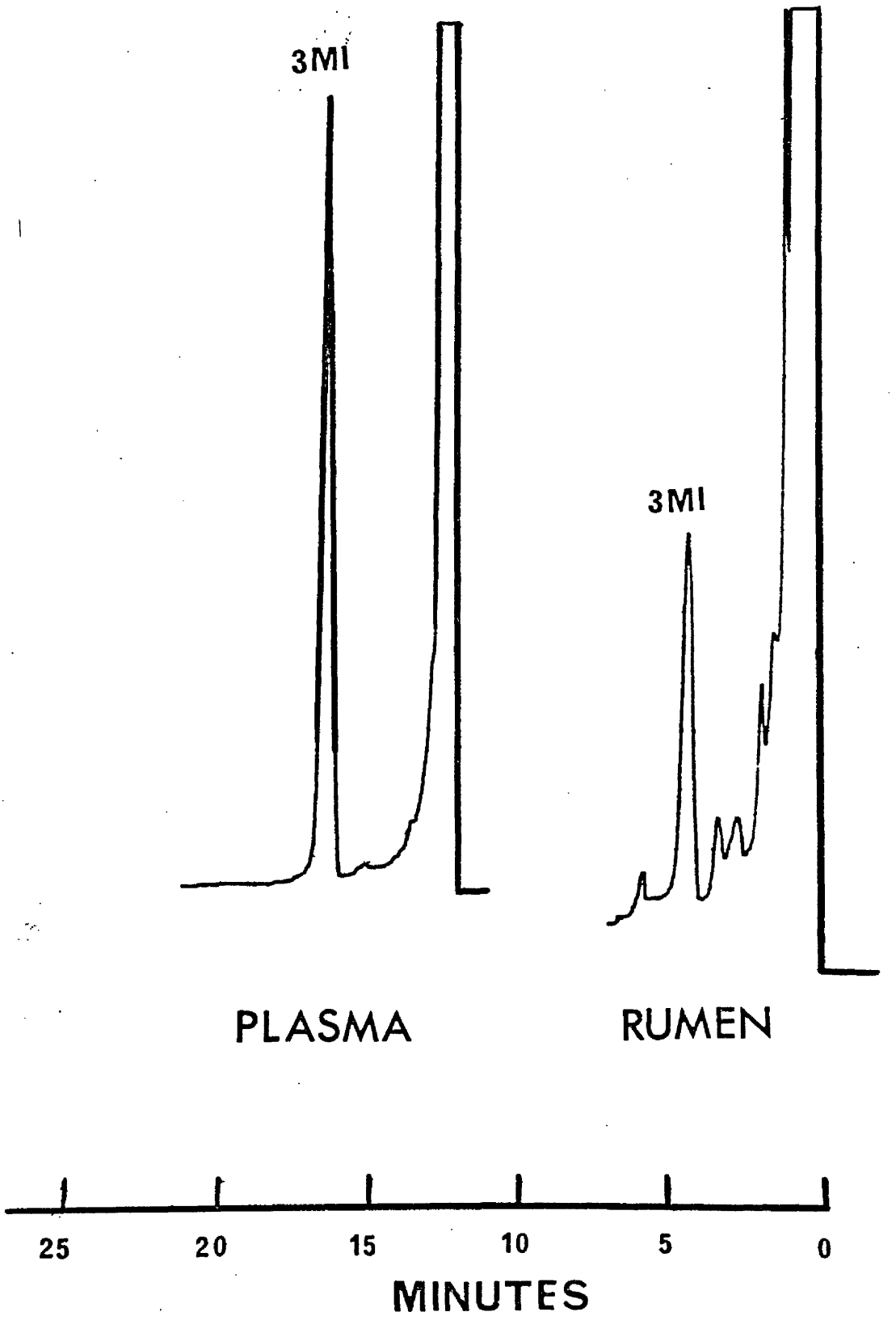

3MI
3MI
PLASMA
RUMEN
25
20
15
10
5
0
MINUTES

TABLE 2.1 RECOVERY OF 3-METHYLINDOLE ADDED TO RUMEN FLUID

3-methylindole added µg	3-methylindole measured µg	Mean ± S.E.	S.E.M. %	Ratio of assayed to added
5	4.8	4.8 ± 0.1	1	0.95
	4.7			
10	9.0	9.3 ± 0.3	3	0.93
	9.5			
20	18.0	18.5 ± 0.5	3	0.93
	19.0			
40	37.0	38.0 ± 1.0	3	0.95
	39.0			

TABLE 2.2 RECOVERY OF 3-METHYLINDOLE ADDED TO BOVINE PLASMA

3-methylindole added µg	3-methylindole measured µg	Mean ± S.E.	S.E.M. %	Ratio of assayed to added
5	4.7	4.7 ± 0.1	1	0.94
	4.6			
10	8.9	9.1 ± 0.2	2	0.91
	9.2			
20	17.1	17.8 ± 0.7	4	0.89
	18.5			
40	35.0	37.0 ± 2.0	5	0.93
	39.0			

2.2.6 - Individual and daily variations in the rate of conversion of L-tryptophan to 3-methylindole by ruminal fluid

The rate of conversion of L-tryptophan to 3-methylindole by samples of fresh ruminal fluid from three cows (designated cows A, B and C) were compared in three experiments. Samples were collected at 10 a.m. on three different days (designated day 1, day 2 and day 3) at ten day intervals. In each experiment duplicate samples (19 ml strained ruminal fluid) from each cow were incubated with L-tryptophan (1 µmol/ml, added in 1 ml 0.1N NaOH) under anaerobic conditions at 37°C in a shaking water bath for 24 hours. Control incubations of fresh ruminal fluid containing no L-tryptophan and boiled ruminal fluid containing L-tryptophan were included. 1 ml samples were taken every six hours for 3-methylindole determination.

Results

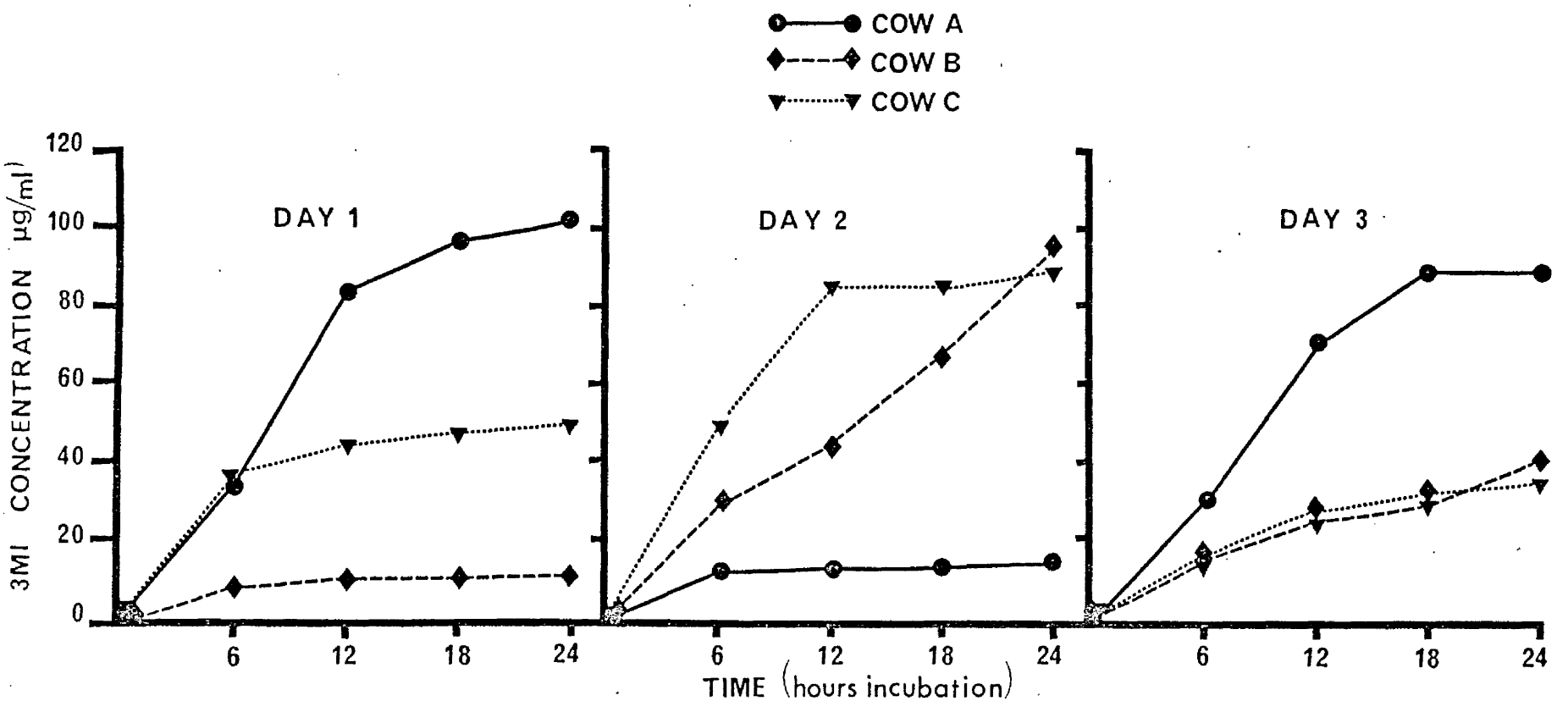
As shown in Fig. 2.4, the rate of production of 3-methylindole from L-tryptophan varied in rumen fluid samples from different cows and in rumen fluid samples from the same cow obtained on different days. The extent of conversion of L-tryptophan to 3-methylindole varied from 8 to 78% in 24 hours incubation.

3-Methylindole production was observed in all control ruminal fluid samples containing no L-tryptophan and never exceeded a concentration of 2 µg/ml after 24 hours incubation.

Indole was detected in incubations of alive ruminal fluid with L-tryptophan in all cases. However it was not detectable in control incubations.

Only traces of 3-methylindole were detectable prior to

Fig. 2.4 Mean 3-methylindole (3MI) concentration in ruminal fluid samples during 24 hours incubation with L-tryptophan (1 µmol/ml). Samples of ruminal fluid were obtained from three fistulated cows (A, B and C) on three different days. Samples were taken at 10 a.m. each day. Each value represents the mean of duplicate samples, the standard error of the mean was small and was less than 5% of the mean in all cases.


COW A
COW B
COW C
3MI CONCENTRATION µg/ml
120
100
80
60
40
20
0
DAY 1
DAY 2
DAY 3
6
12
18
24
TIME (hours incubation)

incubation (<0.2 µg/ml).

2.2.7 - The effect of certain carbohydrates on the conversion of L-tryptophan to 3-methylindole

The effect of different concentrations of glucose, fructose, galactose, mannitol, sucrose, lactose, starch and inulin on the conversion of L-tryptophan to 3-methylindole by ruminal fluid was evaluated in three experiments. These substances are natural constituents of plants and animal food and there are speculations that deficiency of these carbohydrates in grass can be a factor in the aetiology of fog fever (Selman et al, 1976). There are several references indicating that these substances would affect the metabolism of tryptophan and other amino acids by bacterial species (see 2.1.7).

Duplicate samples of strained ruminal fluid (19 ml) were incubated with L-tryptophan (1 µmol/ml, added in 1 ml 0.1N NaOH), and the appropriate carbohydrate (added as a powder). Incubations were done in a shaking water bath at 37°C under an atmosphere of carbon dioxide, as described previously. 1 ml samples of ruminal fluid were taken every six hours for 3-methylindole determination. The pH of ruminal fluid was measured before and after the 24 hours incubation period, and pH changes were measured.

Results

Mannitol (10, 50 and 70 µmol/ml) and starch (2.5, 5 and 10% w/v) caused a decrease in the pH of ruminal fluid with only slight inhibition of 3-methylindole production. Other carbohydrates caused different degrees of inhibition of 3-methylindole production at

Fig. 2.5 The production of 3-methylindole (3MI) in mixtures of ruminal fluid incubated with L-tryptophan (1 µmol/ml) and various carbohydrates or with L-tryptophan alone with no carbohydrate added (control) The concentration of inulin (1) and inulin (2) was 0.5 and 1% (w/v) respectively, the concentration of other carbohydrates was 70 µmol/ml. Each value represented the mean of duplicate samples, the standard error of the mean was small and was less than 8% of the mean in all cases.

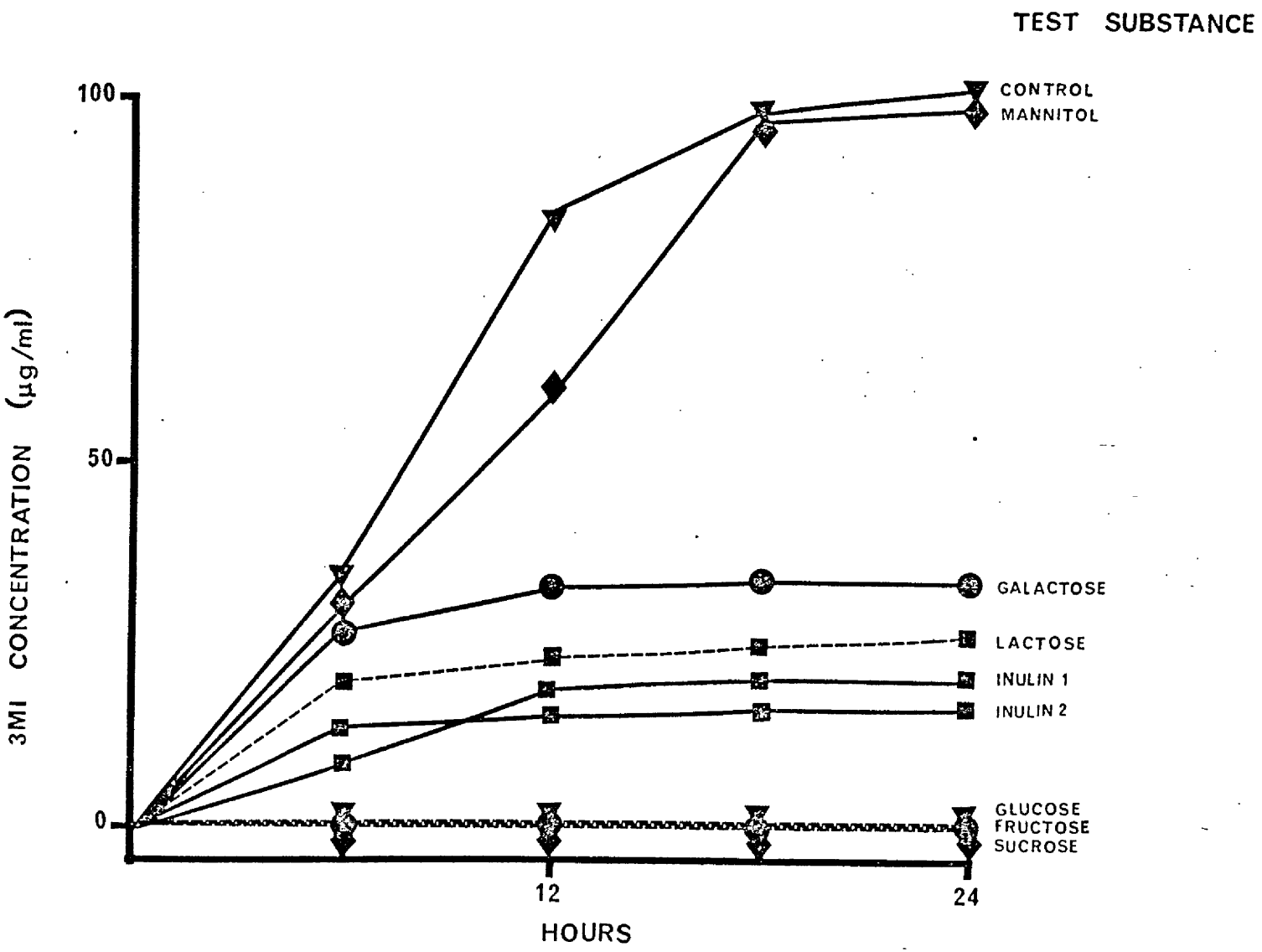

TEST SUBSTANCE
100
50
0
3MI CONCENTRATION (µg/ml)
CONTROL
MANNITOL
GALACTOSE
LACTOSE
INULIN 1
INULIN 2
GLUCOSE
FRUCTOSE
SUCROSE
12
24
HOURS

Fig. 2.6. The production of 3-methylindole (3MI) in mixtures of ruminal fluid incubated with L-tryptophan (1 μmol/ml) and various carbohydrates or with L-tryptophan alone with no carbohydrate added (control). The concentration of starch and inulin was 10% and 2.5% (w/v) respectively, concentration of other carbohydrates was 50 μmol/ml. Each value represents the mean of duplicate samples, the standard error was small and was less than 8% of the mean in all cases.

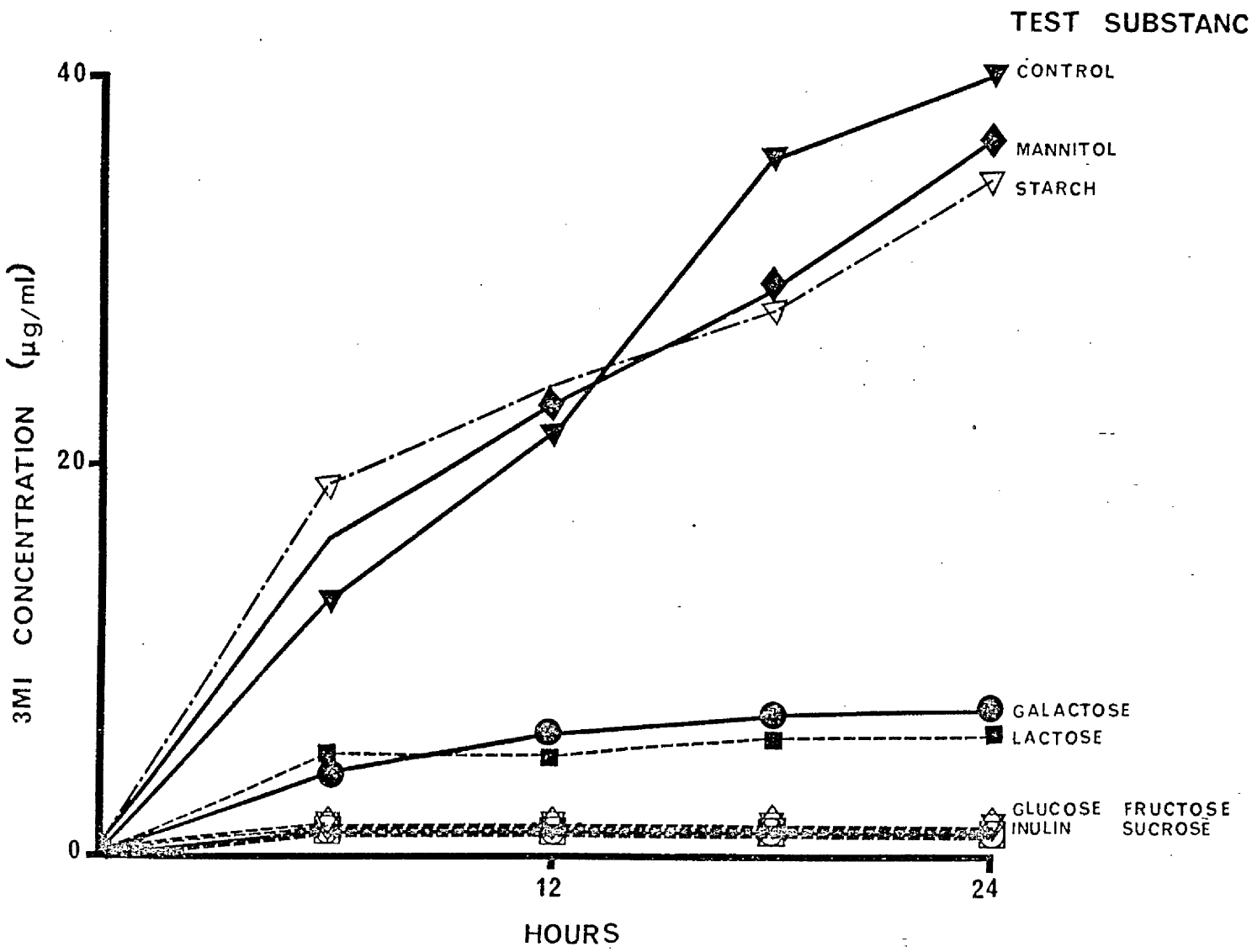

TEST SUBSTANCE
CONTROL
MANNITOL
STARCH
GALACTOSE
LACTOSE
GLUCOSE
INULIN
FRUCTOSE
SUCROSE
40
20
0
3MI CONCENTRATION (µg/ml)
12
24
HOURS

Fig. 2.7 The production of 3-methylindole (3MI) in mixtures of ruminal fluid incubated with L-tryptophan (1 µmol/ml) and various carbohydrates or with L-tryptophan alone with no carbohydrate added (control). Concentration of starch and inulin was 5% (w/v), concentration of other carbohydrates was 10 µmol/ml. Each value represents the mean of duplicate samples. The standard error of the mean was small and was less than 8% of the mean in all cases.

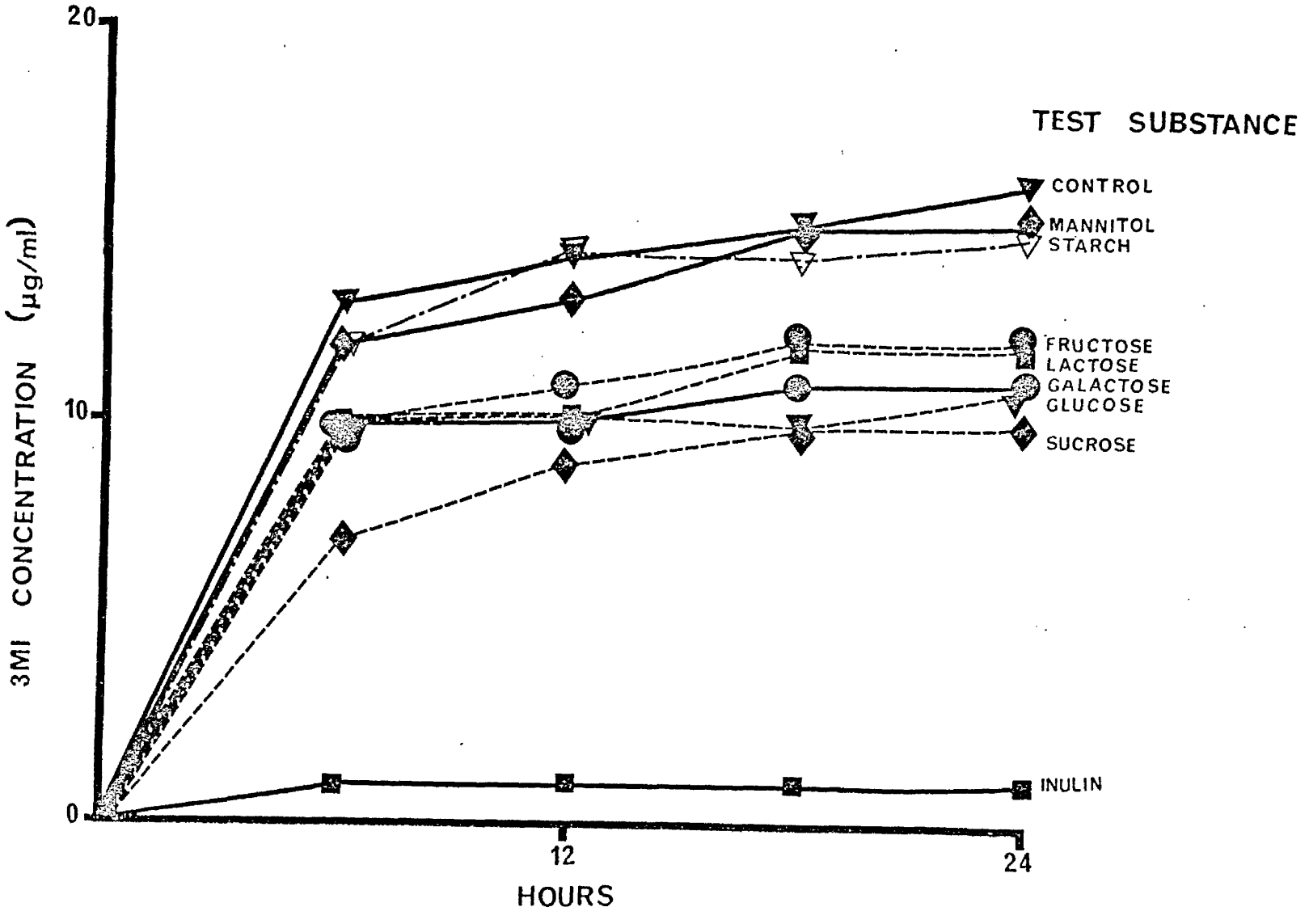

20
10
0
3MI CONCENTRATION (µg/ml)
TEST SUBSTANCE
CONTROL
MANNITOL
STARCH
FRUCTOSE
LACTOSE
GALACTOSE
GLUCOSE
SUCROSE
INULIN
12
24
HOURS

TABLE 2.3 INHIBITION OF 3-METHYLINDOLE PRODUCTION AND DECREASE IN pH IN MIXTURES OF RUMINAL FLUID INCUBATED WITH L-TRYPTOPHAN AND VARIOUS CARBOHYDRATES

Carbohydrate Name	Concentration (µmol/ml)	Decrease in pH (pH units)	% Inhibition
Fructose	10	0.6	25
	50	0.8	100
	70	2.1	100
Glucose	10	0.6	30
	50	0.9	100
	70	2.1	100
Lactose	10	0.6	25
	50	1.4	84
	70	2.4	75
Galactose	10	0.7	30
	50	0.9	83
	70	1.9	68
Sucrose	10	0.6	38
	50	1.6	100
	70	2.6	100
Mannitol	10	0.6	7
	50	0.8	9
	70	1.5	5
	%		
Starch	5	1.1	7
	10	1.3	6
Inulin	0.5	1.5	80
	1	1.6	84
	2.5	1.6	100
	5	1.9	100

different carbohydrate concentrations and a decrease in the pH of ruminal fluid. The effect of different carbohydrates on 3-methylindole production is shown in Figs. 2.5, 2.6 and 2.7. The percentage inhibition of 3-methylindole production and decrease in pH is shown in Table 2.3.

2.2.8 - The effect of certain substances related to carbohydrates on the metabolism of L-tryptophan to 3-methylindole

The effect of different concentrations of trisodium citrate, sodium acetate and sodium lactate and glycerin on the conversion of L-tryptophan to 3-methylindole by ruminal fluid was investigated. These substances are intermediates in carbohydrate metabolism and there are several references indicating that these substances would affect amino acid metabolism by bacterial species (see 2.1.7).

Duplicate samples of strained ruminal fluid (19 ml) were incubated with L-tryptophan (1 µmol/ml, added in 1 ml of 0.1N NaOH), and the appropriate carbohydrate (added as powder) as described previously. 3-Methylindole concentration was determined at the end of the 24 hours incubation period. pH was measured before and at the end of the incubation.

Results

As shown in Table 2.4 only trisodium citrate (100 µmol/ml) caused marked inhibition of 3-methylindole production (100%). A lower concentration of trisodium citrate (25 µmol/ml) caused 15% inhibition of 3-methylindole production. Other substances (sodium lactate, sodium acetate and glycerin) at concentrations of 25 and

100 µmol/ml caused only a slight inhibition of 3-methylindole production (0-3% inhibition). Trisodium citrate, sodium lactate and sodium acetate (25 and 100 µmol/ml) caused a slight rise in pH of ruminal fluid (+ 0.2-1.1 pH units). Glycerin caused a decrease in the pH (- 0.3-0.4 pH units) of ruminal fluid during 24 hours incubation.

2.2.9 - Effect of glucose on the production of 3-methylindole from indoleacetic acid by ruminal fluid

Triplicate samples of strained ruminal fluid (19 ml) were incubated with indoleacetic acid (1 µmol/ml, added in 1 ml 0.1N NaOH) and the appropriate amount of glucose (70 µmol/ml, added as powder). Flasks containing alive ruminal fluid without indoleacetic acid or boiled ruminal fluid with indoleacetic acid were included as controls. Samples of ruminal fluid (1 ml) were taken for 3-methylindole determination. Incubation conditions and 3-methylindole determination were as described previously.

Results

Indoleacetic acid was almost quantitatively converted to 3-methylindole in flasks containing a mixture of alive ruminal fluid and indoleacetic acid (98% conversion). The presence of glucose (70 µmol/ml) inhibited this conversion.

3-Methylindole was detected only in trace amounts in incubation of alive ruminal fluid containing no indoleacetic acid. 3-Methylindole was not detected in incubations of boiled ruminal fluid with indoleacetic acid. Results of the experiment are summarized in Table 2.5.

TABLE 2.4 INHIBITION OF 3-METHYLINDOLE PRODUCTION AND pH CHANGES IN INCUBATION MIXTURES CONTAINING L-TRYPTOPHAN AND VARIOUS SUBSTANCES. VALUES REPRESENT THE MEAN OF TRIPLICATE EXPERIMENTS, S.E.M. WAS LESS THAN 5% OF THE MEAN

Substance Name	Concentration (µmol/ml)	pH change (pH units)	% Inhibition
Trisodium citrate	25	+ 1.0	15
	100	+ 1.1	100
Sodium lactate	25	+ 0.6	2
	100	+ 0.7	1
Sodium acetate	25	+ 0.2	3
	100	+ 0.3	2
Glycerin	25	- 0.3	0
	100	- 0.4	1

TABLE 2.5 PRODUCTION OF 3-METHYLINDOLE IN INCUBATION MIXTURES CONTAINING INDOLEACETIC ACID ALONE OR INDOLEACETIC ACID AND GLUCOSE

Incubation mixture	pH*	3-Methylindole concentration (ug/ml) 6 hours	18 hours	24 hours
Boiled ruminal fluid + indoleacetic acid		0	0	0
Ruminal fluid + distilled water	6.2	2.0 ± 0.1	2.0 ± 0.2	2.0 ± 0.2
Ruminal fluid + indoleacetic acid	6.3	60 ± 0.4	124 ± 3.2	129 ± 0.0
As above + glucose	5.3	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1

* Initial pH = 6.7, values are final pH at the end of incubation.

2.2.10 - The effect of antibiotics and sulphonamides on the conversion of L-tryptophan to 3-methylindole

Three experiments were done to evaluate the inhibitory effect of a number of antibiotics (penicillin, streptomycin, tetracycline, ampicillin and chloramphenicol) and sulfonamides (sulfaguanidine and sulfamethoxypyridazine) on the production of 3-methylindole from L-tryptophan by ruminal fluid. In the first two experiments the appropriate amount of antibiotic or sulfonamide was incubated with ruminal fluid (19 ml) and L-tryptophan (1 µmol/ml, added in 1 ml 0.1N NaOH). In the third experiment the effect of pre-treatment of ruminal fluid with antibiotics was investigated. Ruminal fluid was incubated with antibiotics for 12 hours, L-tryptophan was added (1 µmol/ml, added in 1 ml 0.1N NaOH) and incubation continued for a further 12 hours after which aliquots were taken for 3-methylindole determination. The appropriate amount of antibiotic or sulfonamide was added as a dry powder. Duplicate samples were used. Control flasks with L-tryptophan without antibiotic were included. All incubations were carried out at 37°C in a shaking water bath under a carbon dioxide atmosphere.

Results

Results of the first experiment (Fig. 2.8) showed that chloramphenicol and tetracycline hydrochloride at a concentration of 500 µg/ml caused almost complete inhibition (99%) of 3-methylindole production. Ampicillin and penicillin G sodium (500 µg/ml) caused strong inhibition, whereas sulfaguanidine, sulfamethoxypyridazine and streptomycin sulphate (500 µg/ml) caused only slight inhibition.

Therefore in the second experiment, lower concentrations of chloramphenicol and tetracycline hydrochloride were used (250 µg/ml) and higher concentrations (1 mg/ml) of other antimicrobial agents were used. In this second experiment sulfaguanidine and streptomycin sulphate (1 mg/ml) caused very little inhibition of 3-methylindole production. Sulfamethoxypyridazine (1 mg/ml) caused moderate inhibition of 3-methylindole production. Chloramphenicol, ampicillin, penicillin G sodium and tetracycline hydrochloride (250 µg/ml) caused strong inhibition of 3-methylindole production. Results of this experiment are summarized in Fig. 2.9. Results of the first and second experiments are summarized in Table 2.6.

The first and second experiments (Figs. 2.8 and 2.9) indicate that in the presence of certain antimicrobial agents, in incubation mixtures of alive ruminal fluid with L-tryptophan, most 3-methylindole production occurs during the first twelve hours of incubation, after which 3-methylindole production does not occur to a significant extent, suggesting that antimicrobial agents need to act on ruminal microorganisms, for a period of time, before these antimicrobial agents can inhibit conversion of L-tryptophan to 3-methylindole by ruminal microorganisms. The effect of pre-treatment of ruminal fluid with antimicrobial agents on 3-methylindole production was, therefore, investigated in the third experiment. Concentrations of 50, 100 and 200 µg/ml of those antimicrobial agents which had caused inhibition of 3-methylindole production in the first two experiments were used in this third experiment. Tetracycline hydrochloride pre-treatment was the most effective followed by chloramphenicol.

Fig. 2.8 The production of 3-methylindole (3MI) in mixtures of ruminal fluid incubated with L-tryptophan alone (control) or with L-tryptophan and various antimicrobial agents. Antimicrobial agents were in the form of sulfaguanidine, sulfamethoxypyridazine, ampicillin, penicillin G sodium, chloramphenicol and tetracycline hydrochloride, at a concentration of 500 µg/ml. Each value represents the mean of duplicate samples. The standard error of the mean was less than 8% of the mean in all cases.

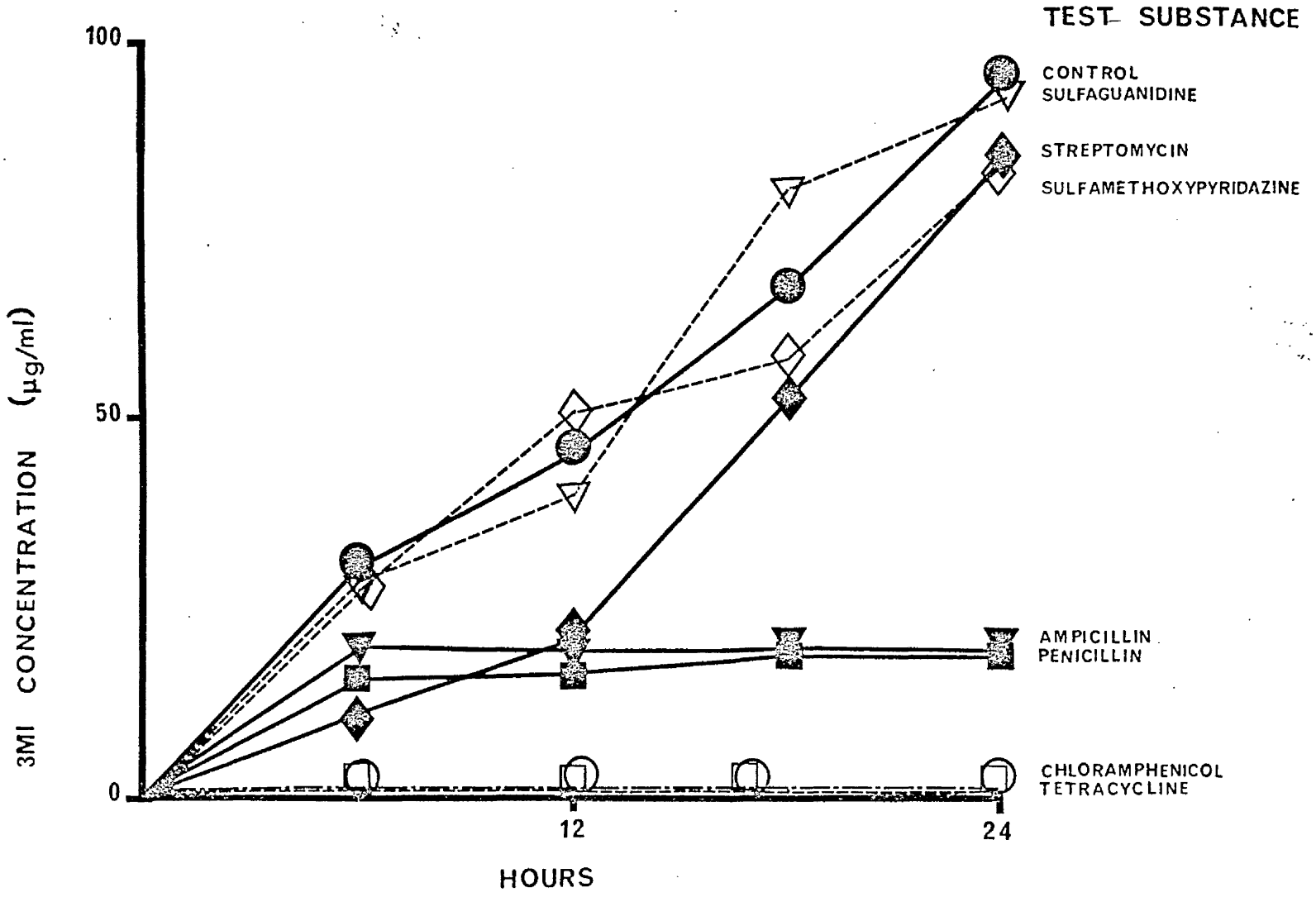

TEST SUBSTANCE
CONTROL
SULFAGUANIDINE
STREPTOMYCIN
SULFAMETHOXYPYRIDAZINE
AMPICILLIN
PENICILLIN
CHLORAMPHENICOL
TETRACYCLINE
100
50
0
3MI CONCENTRATION (µg/ml)
12
24
HOURS

Fig. 2.9 The production of 3-methylindole (3MI) in mixtures of ruminal fluid incubated with L-tryptophan alone (control) or with L-tryptophan and various antibiotics and sulfonamides. Concentration of penicillin G sodium, ampicillin, streptomycin sulphate, sulfaguanidine and sulfamethoxypyridazine was 1 mg/ml and that of tetracycline hydrochloride and chloramphenicol was 250 µg/ml. The standard error of the mean was small and was less than 8% of the mean in all cases.

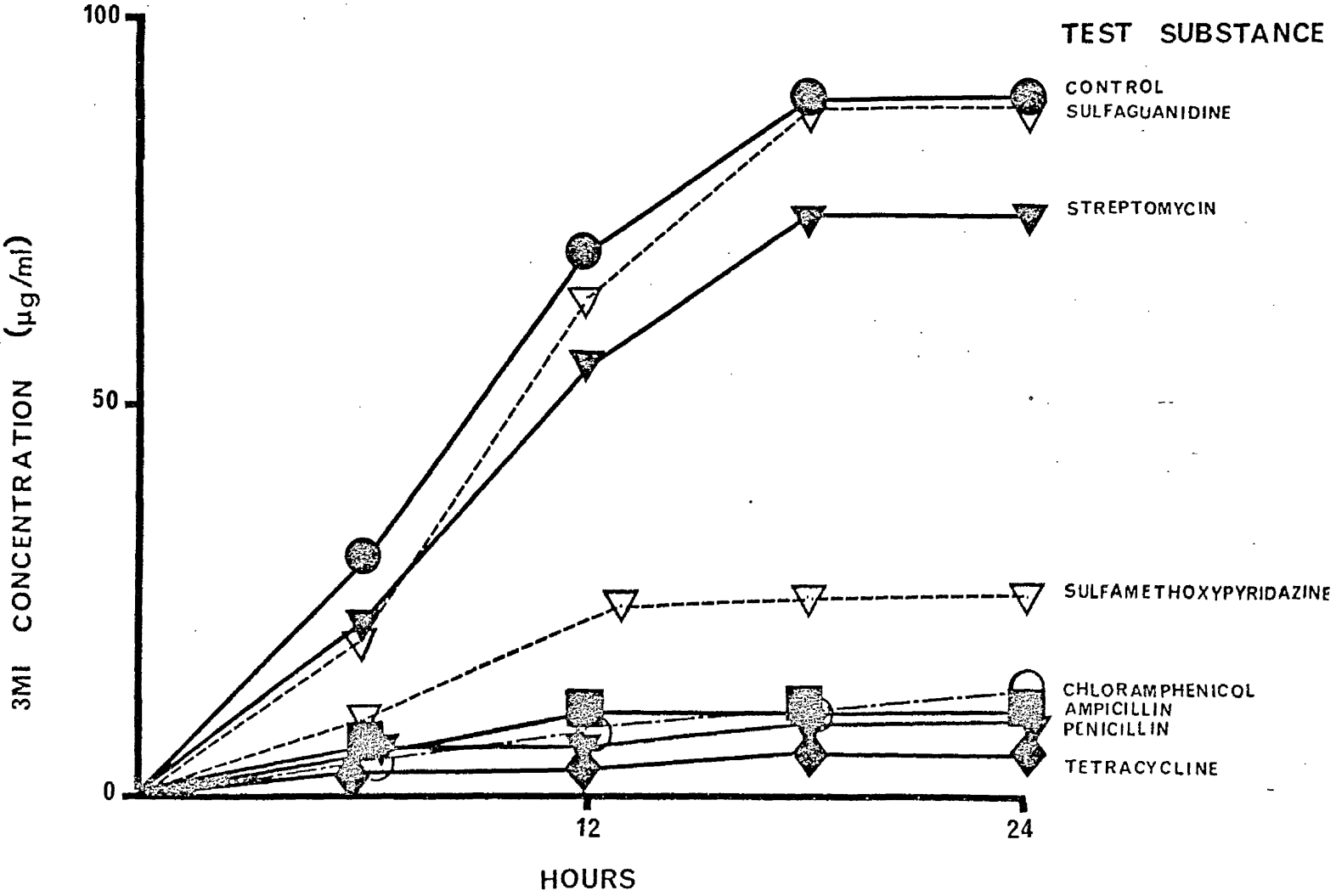

100
50
0
3MI CONCENTRATION (µg/ml)
TEST SUBSTANCE
CONTROL
SULFAGUANIDINE
STREPTOMYCIN
SULFAMETHOXYPYRIDAZINE
CHLORAMPHENICOL
AMPICILLIN
PENICILLIN
TETRACYCLINE
12
24
HOURS

TABLE 2.6 EFFECT OF ANTIBIOTICS (% INHIBITION) ON THE PRODUCTION OF 3-METHYLINDOLE FROM L-TRYPTOPHAN BY RUMINAL FLUID

Antibiotic	Concentration (µg/ml)		
	1000	500	250
Penicillin G sodium	90	81	
Ampicillin	90	81	
Tetracycline hydrochloride		99	94
Chloramphenicol		99	85
Sulfaguanidine	0	2	
Sulfamethoxypyridazine	72	11	
Streptomycin sulphate	16	11	

TABLE 2.7 3-METHYLINDOLE PRODUCTION IN ANTIBIOTIC-PRETREATED RUMINAL FLUID COMPARED WITH NON-ANTIBIOTIC-PRETREATED (CONTROL) RUMINAL FLUID. VALUES REPRESENT THE MEAN OF DUPLICATE EXPERIMENTS ± S.E.M.

Antibiotic Name	Antibiotic Concentration (µg/ml)	3MI Concentration (µg/ml)	% Inhibition of 3MI production
Control		39 ± 0.5	
Chloramphenicol	50	26 ± 1.6	33
	100	16 ± 0.8	57
	200	7 ± 0.0	82
Tetracycline hydrochloride	50	6 ± 0.0	84
	100	0	100
	200	0	100
Penicillin G sodium	50	38 ± 0.5	3
	100	30 ± 0.5	24
	200	18 ± 0.6	45
Ampicillin	50	38 ± 1.6	3
	100	22 ± 1.6	43
	200	18 ± 0.0	55
Sulfamethoxypyridazine	500	38 ± 2.0	3
	750	22 ± 1.8	43
	1000	18 ± 0.0	55

Penicillin G sodium, ampicillin and sulfamethoxypyridazine were less effective in inhibiting 3-methylindole production. The effect of pre-treatment of ruminal fluid with antibacterial agents on 3-methylindole production is shown in Table 2.7.

2.2.11 - Studies on the metabolism of L-(methylene-^{14}C) tryptophan by ruminal fluid in vitro

L-(methylene-^{14}C) tryptophan (specific activity 52 µCi/mmol) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. The compound had a radiochemical purity of 98% as shown by thin layer chromatography in three solvent systems. D-(methylene-^{14}C) tryptophan content by dilution analysis with D-tryptophan was <3%. L-(methylene-^{14}C) tryptophan was diluted with unlabelled L-tryptophan to a specific activity of 62.5 µCi/mmol and stored as a solution (20 µmol/mmol in 0.1N NaOH) at -20°C, and was used within seven days of dilution. The radiochemical purity of the compound was determined at the end of the seven day experimental period using thin layer chromatography on silica gel plates in a solvent system of cyclohexane-chloroform-diethylamine (4:5:1, v/v/v), as described below. There was no change in the radiochemical purity of the compound during the experimental period.

Extraction of metabolites and counting techniques

n-hexane extraction:

A 2 ml sample of ruminal fluid was taken into a centrifuge tube, two drops of 85% phosphoric acid were added to stop further metabolism of radiolabelled tryptophan, and the sample was centrifuged at 2000 rpm for 30 minutes. The supernatant was filtered (Sartorius-

Membran-filter GMBH, pore size 0.45µ), 1 ml of the clear filtrate was taken into an extraction tube and 10 ml n-hexane were added, rotated for 10 minutes and left undisturbed for 5 minutes to separate into lower aqueous and upper organic solvent layers. 1 ml from the hexane and 0.5 ml from the rumen fluid layers were transferred to liquid scintillation vials. .10 ml of the liquid scintillation cocktail ELS93 were added and the radioactivity in the samples counted using a Packard Tri-carb liquid scintillation spectrometer model 3255. Under these conditions counting efficiency was about 70%. All values were corrected for background radioactivity and quenching (internal standard of ^{14}C-toluene).

Extraction of radioactivity from L-(methylene-^{14}C) tryptophan by n-hexane

An amount of radiolabelled tryptophan containing about 100,000 disintegrations per minute (dpm) was made up to 1 ml with ruminal fluid, and subjected immediately to n-hexane extraction. The radioactivity in the hexane and ruminal fluid layers were determined. The distribution of radioactivity between aqueous and organic phases was calculated. Less than 0.2% of the radioactivity partitioned into the hexane layer.

Methylene chloride extraction

Indolic metabolites were extracted using methylene chloride (Perley and Stowe, 1966). The pH of ruminal fluid was adjusted to pH2 with 85% phosphoric acid and extracted with three volumes of methylene chloride to remove the neutral and acidic indolic metabolites. The methylene chloride phase was drawn off. The pH of the ruminal

fluid layer was readjusted to pH8 (saturated potassium carbonate). Basic metabolites were extracted with three volumes of methylene chloride. The methylene chloride fractions were combined and evaporated to 0.2 ml (under nitrogen, 40°C). The concentrated methylene chloride extract was subjected to thin layer chromatography.

Thin layer chromatography of indolic metabolites

Thin layer chromatography of indolic compounds was performed according to the procedure of Haecock and Mahon (1963b) as follows:

Plates: Glass plates (20 cm x 5 cm) were coated with silica gel G. A slurry of silica gel was prepared by mixing 30 g of silica gel and 60 ml distilled water. The slurry was spread (200μ thickness) and left to dry for 10 minutes at room temperature, then at 120°C for one hour and stored in a desiccator.

Solvent systems

Cyclohexane-chloroform-diethylamine (4:5:1, v/v/v) and 1-butanol-acetic acid-water (12:3:5, v/v/v) were used.

Ehrlich's reagent was used to visualize indolic spots. This reagent was prepared by dissolving p-dimethylaminobenzaldehyde (1g) in a mixture of concentrated hydrochloric acid (25 ml) and methanol (75 ml).

The metabolism of L-(methylene-^{14}C) tryptophan by ruminal fluid

Ruminal fluid (19 ml) incubated with L-(methylene-^{14}C) tryptophan (20 μmol of specific activity 62.5 μCi/mmol) added in solution in 1 ml of 0.1N NaOH. Incubation was conducted for 24 hours as described previously. Duplicate samples were taken every six hours. All samples were subjected to the hexane extraction procedure and aliquots of hexane and ruminal fluid were taken for

radioactivity measurement. 10 ml of the combined hexane extract was evaporated to 0.2 ml (under nitrogen) and 10 µl was spotted on silica gel thin layer plates. The plates were developed for one hour in a solvent system of cyclohexane-chloroform-diethylamine (4:5:1, v/v/v). Metabolites were visualized by Ehrlich's spray. The distance between the point of application and the solvent front was divided into twenty equal portions. Each portion was scraped into a separate scintillation vial, 10 ml of the liquid scintillator ELS93 were added and the radioactivity in each vial counted. Authentic samples (10 µl) of indole and 3-methylindole (1 mg/ml, in n-hexane) were run on the same plate.

Samples taken after 12 hours incubation were further examinec using the methylene chloride extraction procedure. Indolic metabolites were extracted using methylene chloride. The combined methylene chloride fractions were evaporated to 0.2 ml. Thin layer chromatography on silica gel thin layer plates in a solvent system of 1-butanol-acetic acid-water (12:3:5, v/v/v) was performed on the concentrated methylene chloride extract as described for the n-hexane extract. Authentic samples of indoleacetic acid and L-tryptophan were dissolved in ethanol (1 mg/ml).

Results:

n-Hexane extraction

As shown in Fig. 2.10 hexane-extractable ^{14}C increased to 62% of the total radioactivity over 24 hours incubation. Total radioactivity decreased and there was a loss of 31% of the radioactivity during the 24 hours incubation. These changes were observed only in

Fig. 2.10 Conversion of L (methylene-^{14}C) tryptophan to hexane extractable metabolites and decrease in total radioactivity during incubation with ruminal fluid. Sample of incubation mixture (1 ml) was extracted with hexane. Radioactivity in hexane (hexane extractable ^{14}C) and in ruminal fluid (hexane non-extractable ^{14}C) was measured). Values represent the mean of duplicate samples. Standard error of the mean was less than 7% in all cases.

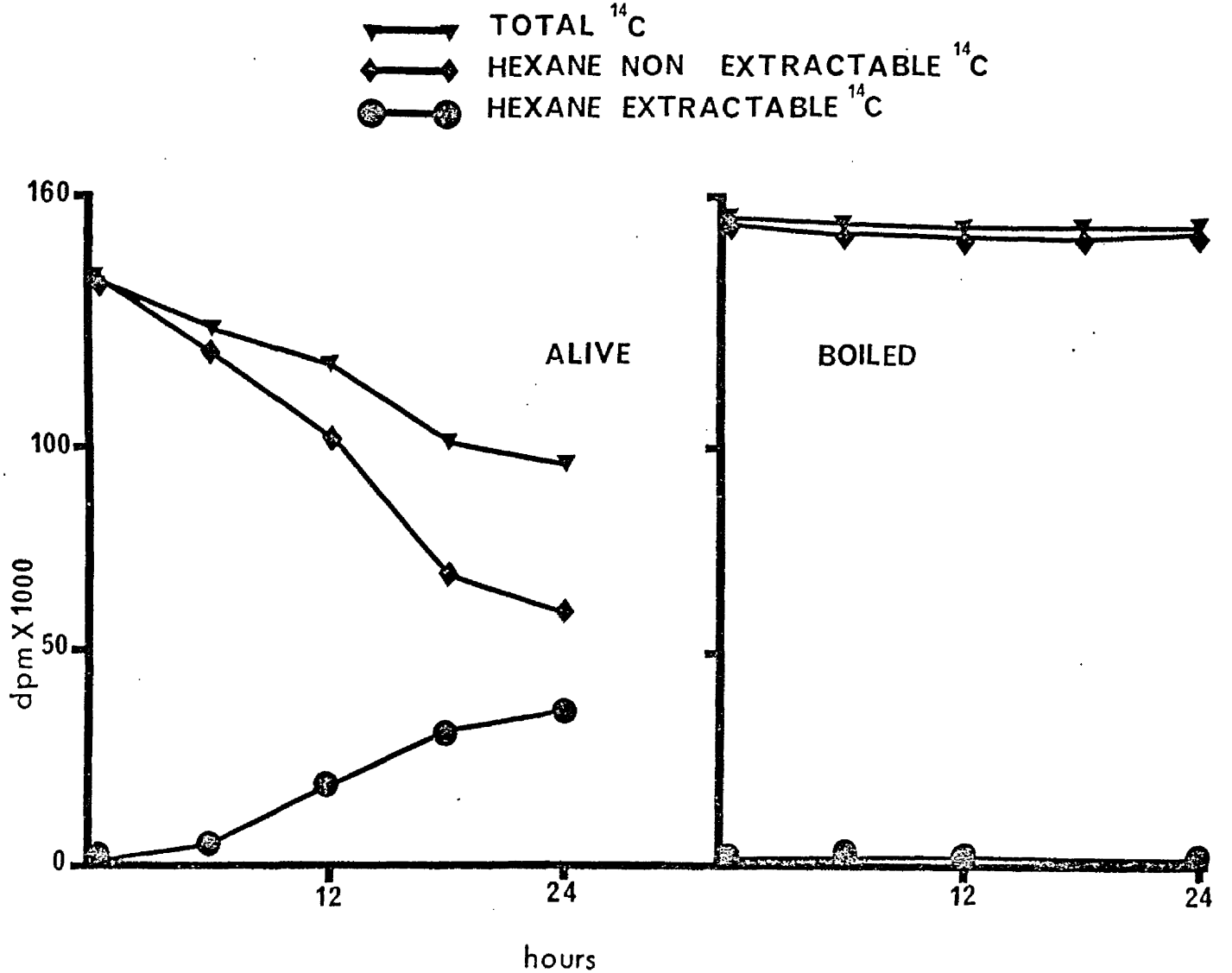

TOTAL ^{14}C
HEXANE NON EXTRACTABLE ^{14}C
HEXANE EXTRACTABLE ^{14}C
160
100
50
0
dpm X 1000
ALIVE
BOILED
12
24
12
24
hours

Fig. 2.11 Hexane extractable L-(methylene-^{14}C) tryptophan metabolites. Ruminal fluid was incubated with L-(methylene-^{14}C) tryptophan. Metabolites were extracted with hexane and separated by thin layer chromatography. The thin layer plate was divided into 20 equal fractions. Fractions were scraped and radioactivity in each fraction measured by liquid scintillation counting.

(3MI = 3-methylindole).

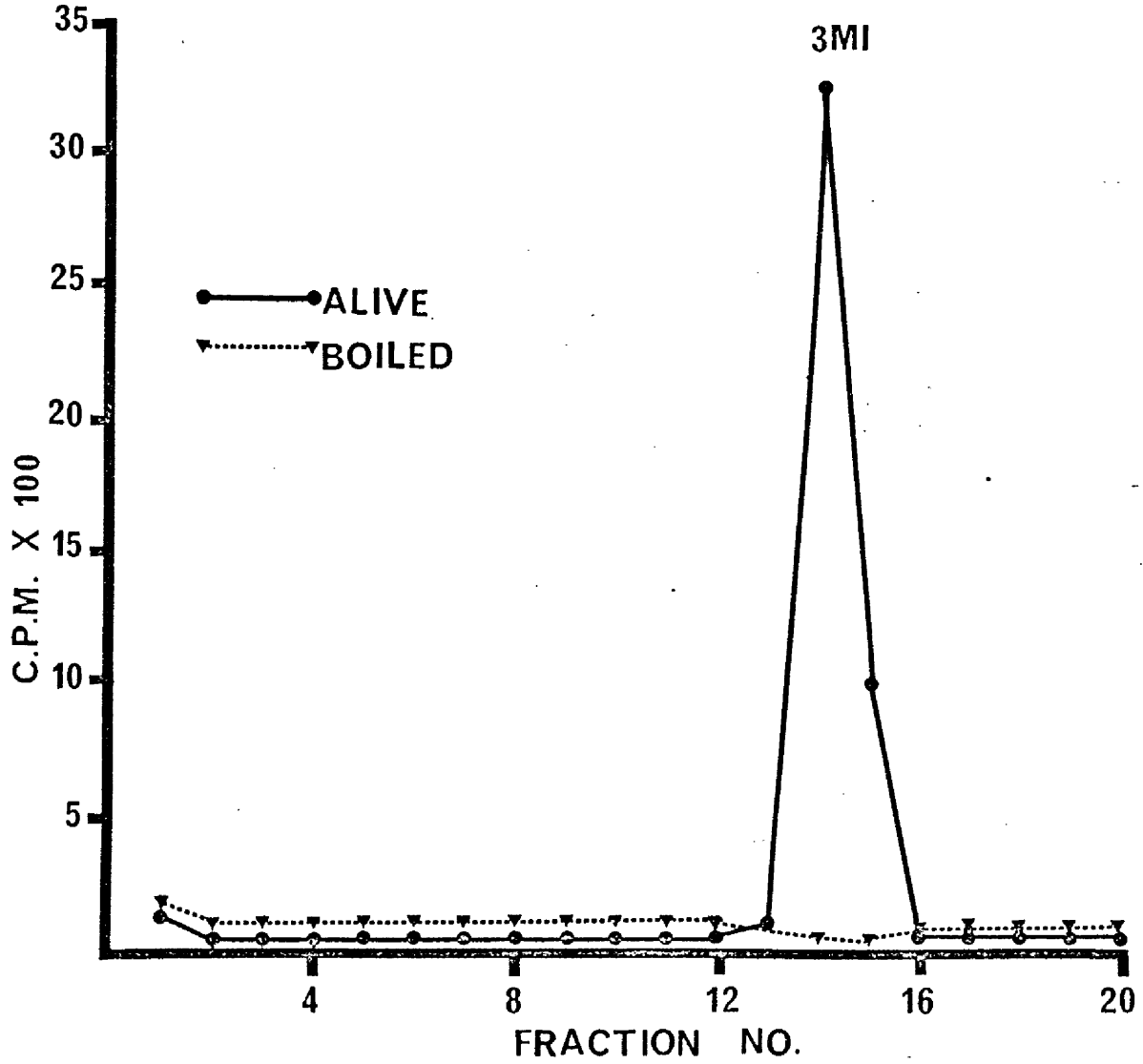
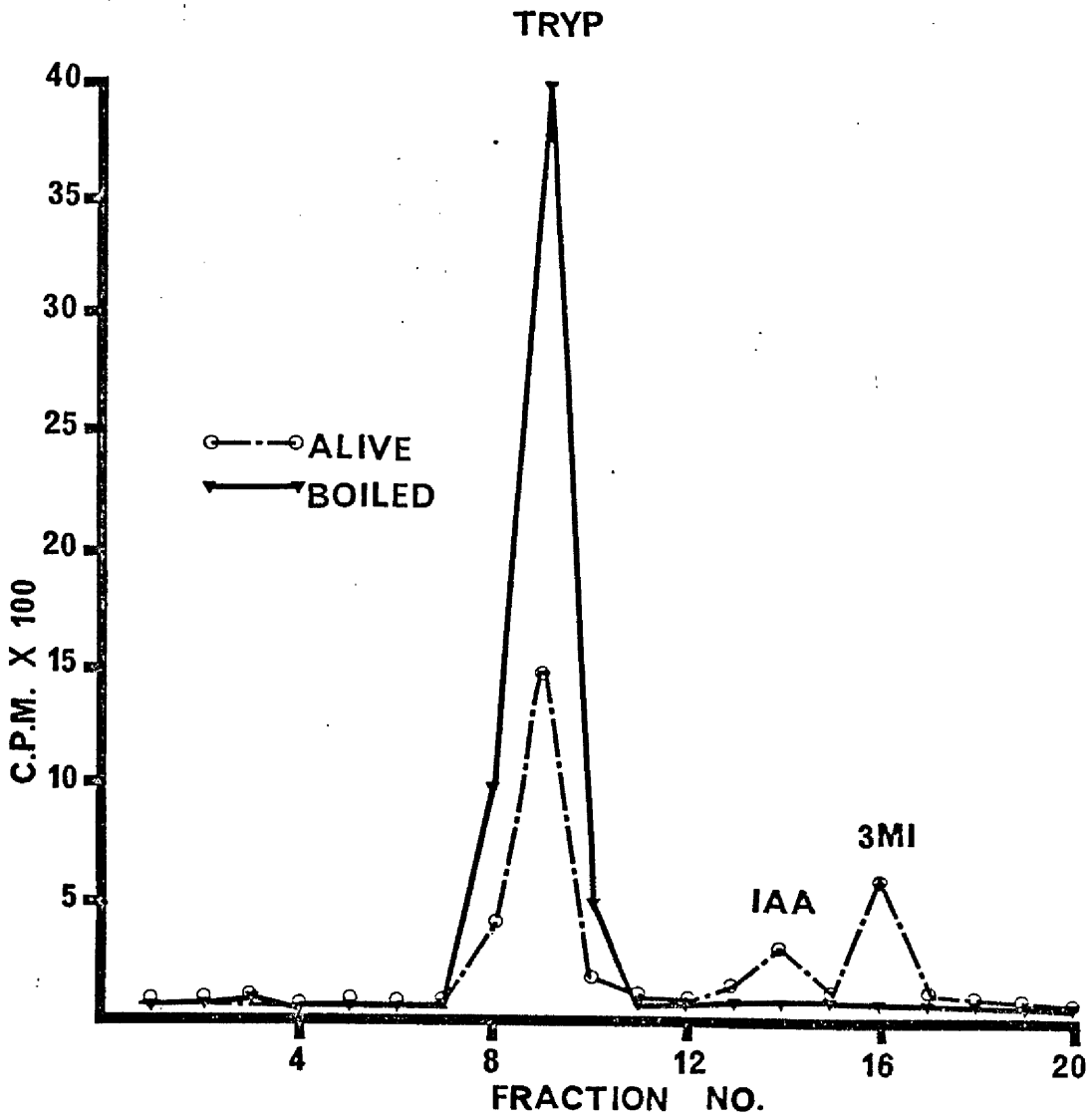

3MI
ALIVE
BOILED
C.P.M. X 100
35
30
25
20
15
10
5
4
8
12
16
20
FRACTION NO.

Fig. 2.12 Methylene chloride extractable L-(methylene-^{14}C) tryptophan metabolites. Ruminal fluid was incubated with L-(methylene-^{14}C) tryptophan. Metabolites were extracted with methylene chloride and separated by thin layer chromotography. The thin layer plate was divided into 20 equal fractions. Fractions were scraped and radioactivity in each fraction measured by liquid scintillation counting.

(TRYP - Tryptophan
IAA = Indoleacetic acid
3MI = 3-methylindole)


TRYP
40
35
30
25
20
15
10
5
C.P.M. X 100
ALIVE
BOILED
3MI
IAA
4
8
12
16
20
FRACTION NO.

incubation flasks containing alive ruminal fluid. On thin layer plates there were two spots corresponding to indole and 3-methylindole with Ehrlich's reagent, 3-methylindole gave a violet coloured spot (RF value 0.7) and indole gave a red spot (RF value 0.6). As shown in Fig. 2.11 more than 90% of the radioactivity extracted by n-hexane appeared in the 3-methylindole spot.

Methylene chloride extraction

The methylene chloride extract of boiled rumen fluid gave only one spot with RF value (RF = 0.45) and colour reaction (purple) similar to an authentic tryptophan sample.

Methylene chloride extract of ruminal fluid gave three spots, one purple spot identical to tryptophan (RF = 0.45) and two overlapping spots, one purple (RF = 0.7) identical to indoleacetic acid and one violet spot (RF = 0.78) identical to 3-methylindole. Typical radioactivity in different thin layer chromatoplate fractions is shown in Fig. 2.12.

2.2.12 - Effect of 3-methylindole on the rate of conversion of L-(methylene-^{14}C) tryptophan to (methyl-^{14}C) 3-methylindole by ruminal fluid

This experiment was conducted to investigate the effect of the presence of 3-methylindole (10 and 100 µg/ml) on the extent of metabolism of L-tryptophan to 3-methylindole. 20 µmol of L-(methylene-^{14}C) tryptophan (specific activity 62.5 µCi/mmol) dissolved in 1 ml 0.1N NaOH and the appropriate amount of 3-methylindole dissolved in 0.5 ml of 10% "Cremophor-EL" were added to 19 ml strained ruminal fluid and incubated as described previously. In control flasks, only

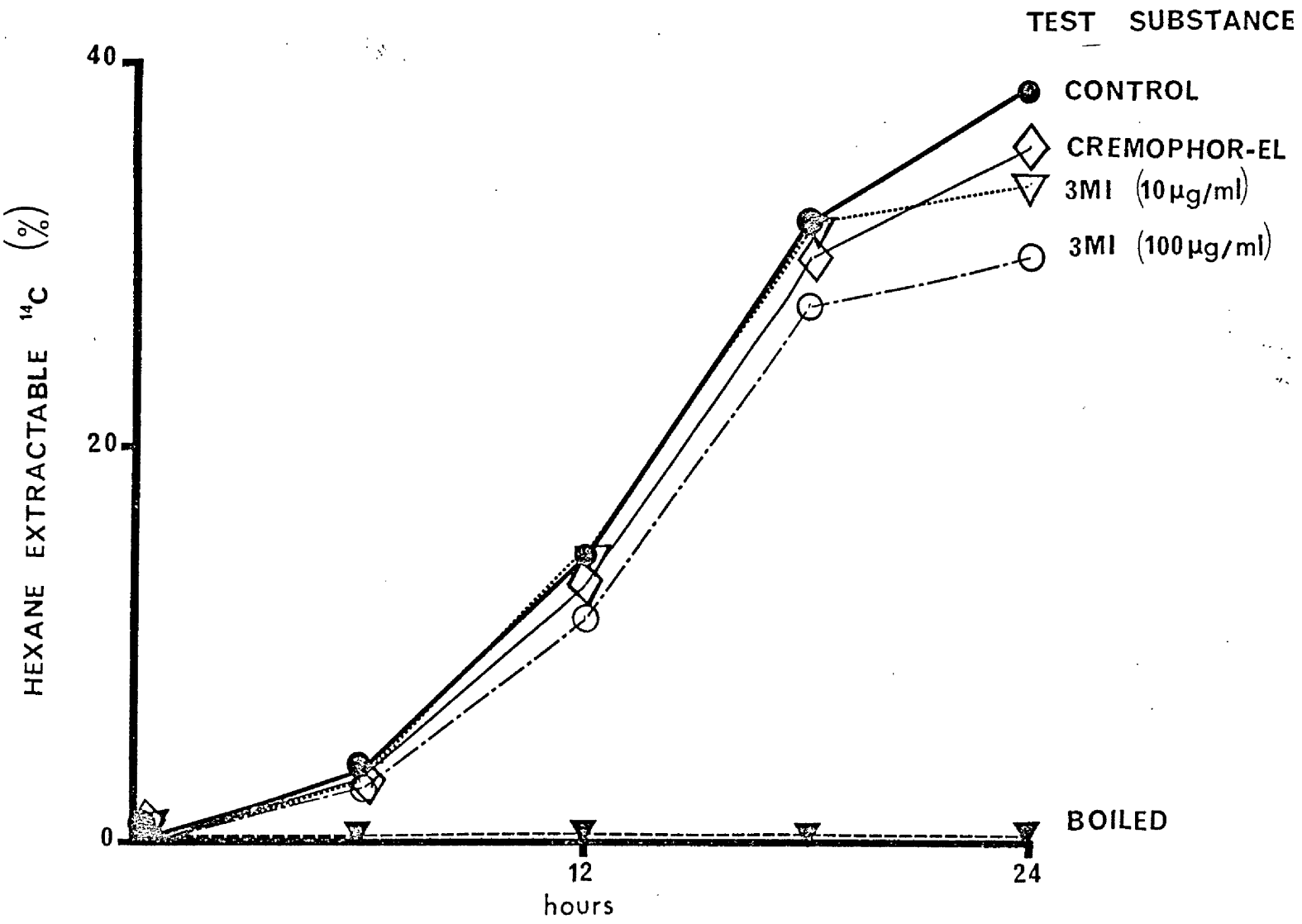
the vehicle of 3-methylindole or distilled water was added. Boiled ruminal fluid controls were also included. 1 ml samples were taken every six hours.

As the difference in concentration of 3-methylindole added to different incubation flasks would affect the efficiency of 3-methylindole extraction, 0.2 ml of 5% Cremophor-EL in distilled water, containing the appropriate amount of 3-methylindole was added to each sample so that the final concentration of 3-methylindole in each sample was 100 µg/ml + 3-methylindole produced during incubation. All samples were subjected to the hexane extraction procedure. Hexane extractable ^{14}C was calculated as a percentage of total radioactivity in both the aqueous and hexane layers. Previous results (Fig. 2.11) indicate that the hexane extraction procedure is specific for 3-methylindole. More than 90% of radioactivity extracted in n-hexane appeared in the 3-methylindole spot.

Results:

As shown in Fig. 2.13 hexane extractable ^{14}C increased to a maximum of 38.2% of total radioactivity over 24 hours incubation in mixtures containing alive ruminal fluid, radiolabelled tryptophan and distilled water. In incubation mixtures containing Cremophor-EL or 3-methylindole at concentrations of 10 or 100 µg/ml instead of distilled water the hexane extractable ^{14}C reached a maximum of 35.5, 33.3 and 29.8% of the total radioactivity respectively. These results indicate that concentrations of 10 and 100 µg/ml of 3-methylindole caused 13 and 22% inhibition of 3-methylindole production respectively. The vehicle of 3-methylindole, Cremophor-EL, caused 7% inhibition of

Fig. 2.13 The effect of Cremophor-EL and 3-methylindole (3MI) on the conversion of L-(methylene-^{14}C) tryptophan to hexane extractable metabolites by ruminal fluid. Incubation mixtures contained alive ruminal fluid, radiolabelled tryptophan and distilled water (control) or Cremophor-EL or a solution of 3-methylindole in Cremophor-EL. Incubation of boiled ruminal fluid (boiled) contained boiled ruminal fluid, radiolabelled tryptophan and distilled water. Incubation mixture was extracted with hexane. Radioactivity was measured in hexane and ruminal fluid layers. Radioactivity in hexane was expressed as percentage of the total radioactivity (% hexane extractable ^{14}C).


TEST SUBSTANCE
CONTROL
CREMOPHOR-EL
3MI (10µg/ml)
3MI (100µg/ml)
BOILED
HEXANE EXTRACTABLE ^{14}C (%)
40
20
0
12
24
hours

3-methylindole production.

2.2.13 - The effect of oral dosing with L-tryptophan in cattle

In this experiment adult (more than three years old) Hereford and Hereford cross cows were used. Animals were clinically normal prior to the start of the experiment. Hay and water were freely offered to all cattle during the experimental period. Cattle were fed poor quality hay ad libitum but no concentrates for six weeks prior to dosing with L-tryptophan. Four test (500-600 kg body weight) cattle were housed for five days before dosing until the end of the experiment. L-tryptophan (0.5 g/kg) was administered through a stomach tube as a suspension in five litres of water; control cattle each received five litres of water by the same route.

Respiratory rates recorded here were obtained during the daily clinical examination which was made at 10.00 hours from the day prior to dosing for eight days. Observations were carried out several times daily over this period.

Heparinized blood samples were collected at two hour intervals for 36 hours and then at 44, 48, 54 and 72 hours after tryptophan dosing. Plasma was separated and stored at -20^{o}C until analysis for 3-methylindole and tryptophan. Analysis for 3-methylindole was conducted as described previously.

Analysis for tryptophan was made within 24 hours after collecting the sample, as described below.

Assay of tryptophan in bovine plasma

Tryptophan was determined in bovine plasma using a procedure based on a method for spectrofluorometric determination of tryptophan

in human serum, by Guilbault and Froehlich (1973). This assay is based on the facile reaction of tryptophan with aqueous formaldehyde. The condensation products have a lower fluorescence quantum yield and so the concentration of the amino acid can be measured by the reduction in fluorescence as the reaction proceeds.

Reagents

The following reagents were used:

1) Trichloroacetic acid solution: 10% trichloroacetic acid (w/v) in distilled water.
2) Formaldehyde reagent: two volumes of commercial formaldehyde solution (37-40%) diluted with one volume of water. The pH was adjusted to 7.3 using 0.1N NaOH and the reagent filtered.
3) 5% sodium bicarbonate solution in water (w/v).
4) Distilled water adjusted to pH 7.3 using 0.1N NaOH.

Procedures

1 ml of plasma was mixed with 1 ml of water and 5 ml of trichloroacetic acid solution (10%). The mixture was shaken for five minutes to ensure total deproteinization. The precipitate was removed by centrifugation (2000 rpm for 30 minutes). 2 ml of the supernatant liquid were pipetted and transferred to a graduated test tube (10 ml capacity) and 3 ml of 5% sodium bicarbonate solution were added dropwise (to prevent loss of the sample due to the violent effervescence) with frequent shaking. The pH of the sample was measured. If the pH was less than 7.3, additional sodium bicarbonate was added to adjust the pH to 7.3-7.4. Water (pH 7.3) was added to make up the sample volume to 10 ml.

1.5 ml were pipetted into the spectrofluorimeter cuvette (4 ml capacity). The following steps were accurately timed using a laboratory stop watch, in the following sequence:

Time (seconds)

0: 1.5 ml formaldehyde reagent were added to the sample in the cuvatte.

11 to 15: The cuvette was placed in the spectrofluorimeter.

16 to 30: The spectrofluorimeter reading stabilized over this period and was read at 30 seconds.

30 to 90: The decrease in the spectrofluorimeter reading was recorded over this period.

The spectrofluorimeter (Farrand spectrofluorimeter MK-1, Farrand Optical Co. Inc., New York) was set at 280 µm excitation and 360 µm emission wavelength.

Standards

A series of 12 tryptophan solutions, ranging in concentration from 1-200 µg/ml were prepared in distilled water and treated as described in the assay procedure. The change in fluorescence was plotted against the concentration.

Blank

A blank of water was run with no change in the signal from the spectrofluorimeter over a period of three minutes.

Results:

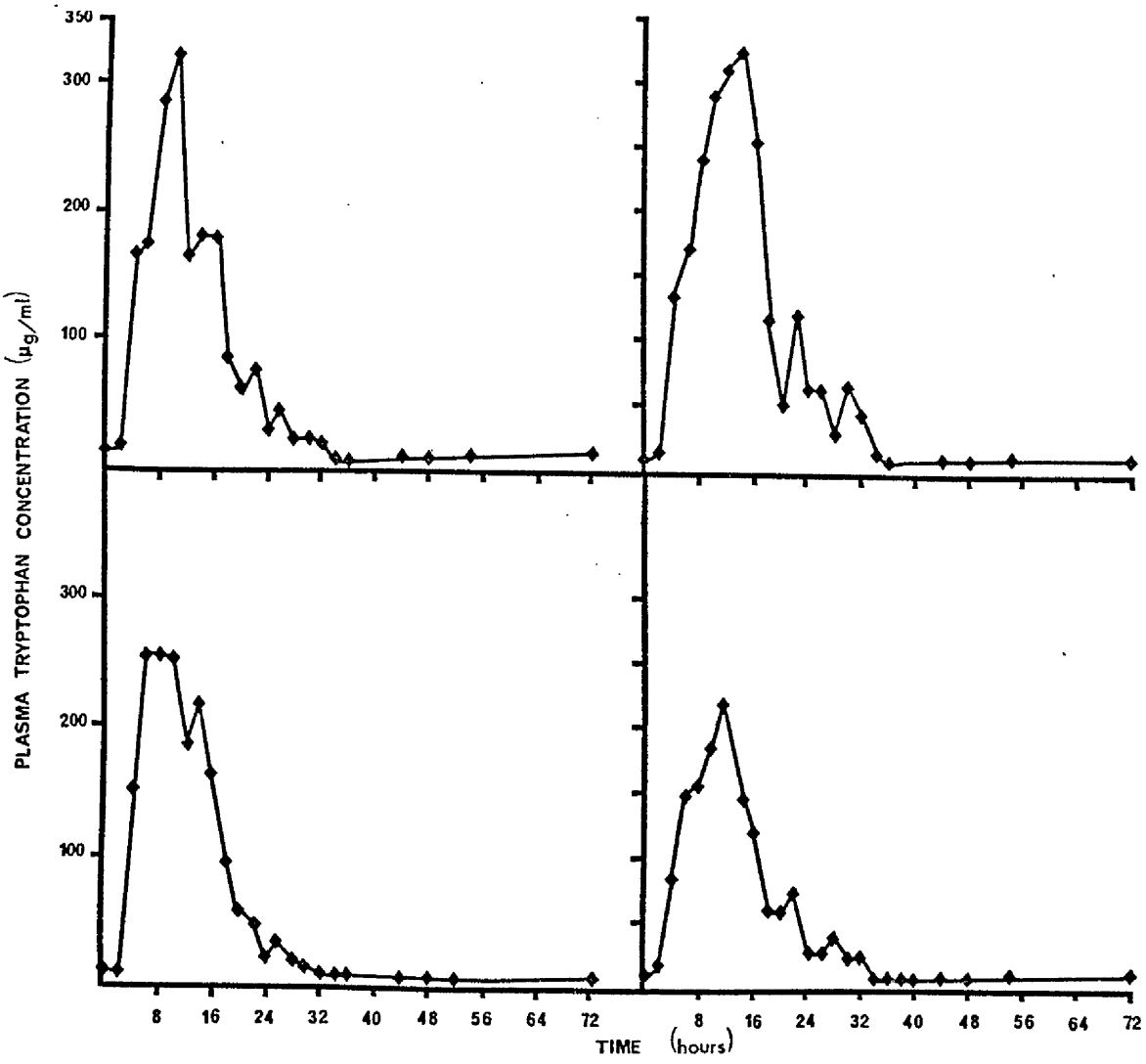
Clinical findings

No obvious depression was noted in the animals after dosing. Day-to-day changes in respiratory rate were minimal and no significant

TABLE 2.8 RESPIRATORY RATES OF CATTLE AFTER ORAL ADMINISTRATION OF L-TRYPTOPHAN IN CATTLE

	L-tryptophan dosed cows				Control cows	
Day	A	B	C	D	E	F
0	24	24	24	30	30	30
1	24	24	24	30	30	30
2	30	24	24	30	30	30
3	24	30	24	36	24	24
4	24	24	24	30	24	24
5	30	24	24	30	30	30
6	30	24	24	30	30	30
7	24	24	24	30	30	30
8	24	24	24	30	30	30

Fig. 2.14 Concentration of tryptophan in plasma of four cows given an oral dose of 0.5 g/kg body weight of L-tryptophan


PLASMA TRYPTOPHAN CONCENTRATION (µg/ml)
350
300
200
100
300
200
100
8
16
24
32
40
48
56
64
72
8
16
24
32
40
48
56
64
72
TIME (hours)

increase was detected in any of the test cattle. Respiratory rates of test and control cattle are shown in Table 2.8.

Concentration of 3-methylindole in plasma

3-Methylindole was not detectable in any of the plasma samples. The limit of detection of the 3-methylindole assay was 0.01 µg/ml.

Concentration of tryptophan in plasma

Plasma tryptophan concentration rose gradually. Maximal concentrations in plasma were reached at 14 hours after tryptophan administration. Plasma concentrations of tryptophan returned to pre-administration levels 34 hours after oral administration of L-tryptophan. Plasma tryptophan concentrations are shown in Fig. 2.14.

2.3 - DISCUSSION

It has previously been established that oral administration of tryptophan produces a disease similar to fog fever in cattle (Dickinson et al, 1967; Dickinson, 1970; Dickinson and Piper, 1971; Yokoyama et al, 1975). The parent compound L-tryptophan has no pulmonary toxic effect (Carlson et al, 1968); its ruminal metabolite 3-methylindole is probably responsible for the pulmonary toxic effects of orally administered L-tryptophan (Carlson et al, 1972; Yokoyama and Carlson, 1974; Yokoyama et al, 1975). Production of abnormal amounts of 3-methylindole have been associated with both the naturally occurring and the L-tryptophan induced pulmonary disease (Yokoyama et al, 1975; Terry et al, 1976). The response of cattle to sudden change of pasture and to orally administered L-tryptophan is extremely variable (Dickinson et al, 1967; Dickinson, 1970; Dickinson and Piper, 1971;

Selman et al, 1974; Yokoyama et al, 1975). Results of the present work strongly suggest that these variations arise from variations in the rate of conversion of L-tryptophan to its metabolite 3-methylindole by ruminal microorganisms. Samples of ruminal fluid from different animals or from the same animal on different days displayed marked differences in their ability to convert L-tryptophan to 3-methylindole. The extent of conversion of tryptophan varied from 8 to 87% conversion to 3-methylindole in different samples of ruminal fluid after a period of 24 hours of incubation (Fig. 2.4.).

A dose of 0.35 g/kg of L-tryptophan given orally has been used previously to induce pulmonary disease. This dose usually results in the development of severe respiratory distress and/or death in approximately 50% of cows within four days after dosing (Carlson et al, 1972; Yokoyama et al, 1975; Hammond, Carlson and Breeze, 1978; Hammond, Bray, Cummins, Carlson and Bradley, 1978). In this present study a dose of 0.5 g/kg of L-tryptophan did not cause respiratory distress in any of four cows. This indicates that within certain dose limits the incidence and severity of pulmonary disease is not necessarily dependent on the size of the dose.

After oral administration of L-tryptophan (0.5 g/kg body weight) 3-methylindole was not detected in plasma of any of the four cows. An increase in L-tryptophan concentration was detected in the plasma of these cows. Maximum concentrations of L-tryptophan in plasma were reached 6-14 hours after administration of L-tryptophan given orally and returned to pre-administration concentrations 32-36 hours after oral administration of L-tryptophan (Fig. 2.14). Thus failure

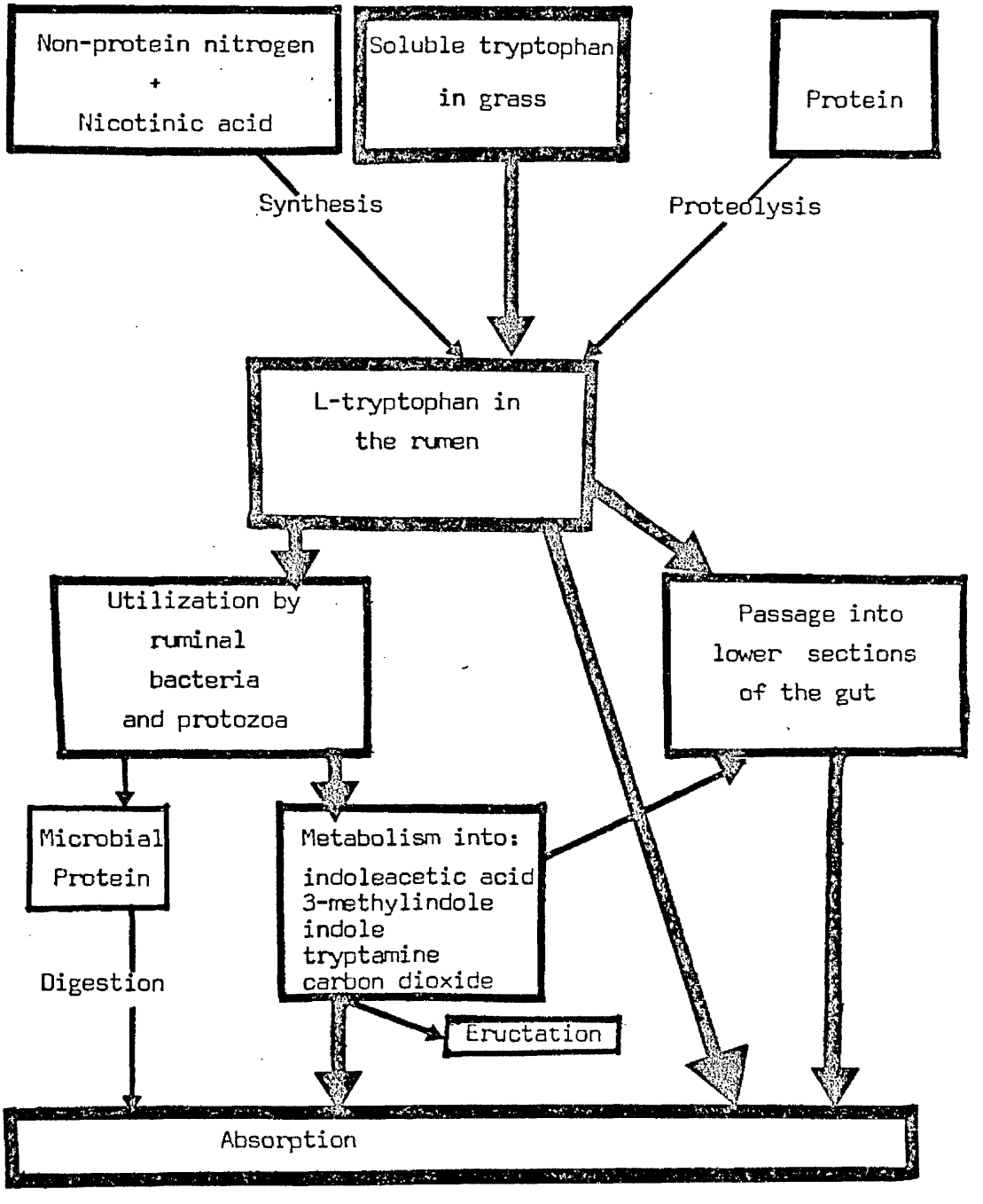
of L-tryptophan to induce pulmonary distress was due to failure of ruminal microorganisms to convert L-tryptophan to 3-methylindole.

The site of absorption of tryptophan, in cattle, is not known. Studies in sheep indicate that absorption of tryptophan occurs from the rumen and reticulum at significant rates (Candlish, Stranger, Devlin and La Croix, 1970).

Results of this present study and of other studies (Lewis and Emery, 1962a, b, c; Scott et al, 1963; Carlson et al, 1968; Schatzman and Gerber, 1972; Yang and Carlson, 1972; Yokoyama and Carlson, 1974) indicate that the fate of L-tryptophan in cattle following oral administration involves several competing pathways. Tryptophan can be available for: absorption and utilization by the animal body; utilization and incorporation into ruminal microorganisms, probably for synthesis of microbial protein; or its metabolism into other compounds including 3-methylindole. The amounts of 3-methylindole formed would depend on the relative rates of different metabolic routes for L-tryptophan and on factors directly affecting the metabolic pathway leading to 3-methylindole formation. Possible sources and metabolic fates of L-tryptophan in the rumen of cattle are summarized in Fig. 2.15.

Mackenzie et al (1975) have failed to identify a difference between the tryptophan content of normal pasture and pasture inducing fog fever in Britain. These observations do not exclude the possibility that tryptophan is the fog fever inducing agent in this pasture, since enhanced conversion of L-tryptophan to 3-methylindole, rather than the intake of abnormal amounts of the amino acid, may be responsible for the

Fig. 2.15 Fate of tryptophan after oral administration in cattle


Non-protein nitrogen
+
Nicotinic acid
Soluble tryptophan
in grass
Protein
Synthesis
Proteolysis
L-tryptophan in
the rumen
Utilization by
ruminal
bacteria
and protozoa
Passage into
lower sections
of the gut
Microbial
Protein
Metabolism into:
indoleacetic acid
3-methylindole
indole
tryptamine
carbon dioxide
Digestion
Eructation
Absorption

disease.

Previous studies have been centred on the tryptophan content of grass as a possible precursor of 3-methylindole (Mackenzie et al, 1975; Selman et al, 1977). These studies ignored other possible indolic substrates convertible to 3-methylindole. In this present study indoleacetic acid, which is a natural plant constituent (Meister, 1965) was almost quantatively (98.5%) converted to 3-methylindole by ruminal fluid (Table 2.5). Ruminal fluid can also convertindole pyruvate, indoleacetaldehyde and indolealdehyde to 3-methylindole (Yokoyama and Carlson, 1974). It would appear however that the amounts of these three other possible precursors are also insufficient in grass to account for the concentrations of 3-methylindole required.

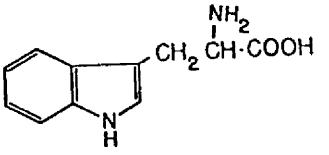
Apart from dietary sources of soluble tryptophan, tryptophan can also be synthesized by ruminal microorganisms from non-protein nitrogenous substances in the presence of nicotinic acid (Piana and Piva, 1969). It would also be possible to produce soluble tryptophan from dietary protein by enzymatic hydrolysis, since ruminal microorganisms possess proteolytic activity (Warner, 1956).

Studies with radiolabelled tryptophan indicate that there was a continuous loss of radioactivity during incubation of L-(methylene -^{14}C) tryptophan with ruminal fluid. 31% of the total radioactivity was lost during 24 hours incubation (Fig. 2.10).

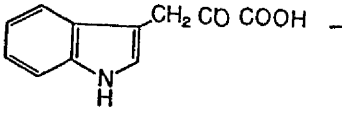
This confirms a previously published observation by Yokoyama and Carlson (1974) who proposed that loss of radioactivity was possibly due to complete degradation of the labelled tryptophan to carbon dioxide. Radio-thin layer chromatographic studies indicate that indoleacetic acid and 3-methylindole were formed from L-tryptophan by ruminal microorganisms (Figs. 2.11 and 2.12). Indole production was detected by gas-liquid chrometography in incubations of L-tryptophan with ruminal fluid. Indoleacetic acid was converted to 3-methylindole (Table 2.5). These results are in a good agreement with the findings of Yokoyama and Carlson (1974) who proposed that indoleacetic acid is formed from L-tryptophan as an intermediate between L-tryptophan and 3-methylindole. The putative metabolic route leading to the formation of 3-methylindole from L-tryptophan is shown in Fig. 2.16. 3-Methylindole at a concentration of 10 µg/ml and 100 µg/ml caused 13 and 22% inhibition, respectively of 3-methylindole production from labelled L-tryptophan. Cremophor-EL, the vehicle used for solubilizing 3-methylindole, caused 7% inhibition of 3-methylindole production (Fig. 2.13). The hexane extraction procedure used to quantify 3-methylindole production in experiments using radiolabelled tryptophan (Figs. 2.10 and 2.13) was specific for 3-methylindole. More than

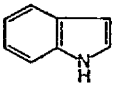
Fig. 2.16 Putative metabolic route of tryptophan to 3-methylindole in the rumen of cattle

L-tryptophan



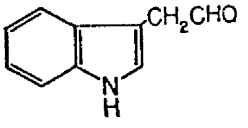
Indolepyruvic acid



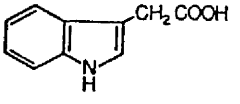


Indole

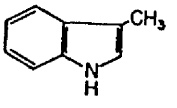
Indoleacetaldehyde



Indole-3-acetic acid



3-methylindole



90% of radioactivity extractable by hexane appeared in the 3-methylindole spot using thin layer chromatography (Fig. 2.11). Indole did not interfere with the procedure as the methylene ^{14}C of tryptophan is lost. Therefore hexane extractable ^{14}C (Figs. 2.10 and 2.13) represented 3-methylindole.

A previous study (Yokoyama and Carlson, 1974) showed that glucose can inhibit the conversion of L-tryptophan to 3-methylindole (in vitro). In this present study the inhibition of 3-methylindole production by glucose has been shown not to be specific to this sugar but shared by many carbohydrates (Figs. 2.5, 2.6 and 2.7) and substances related to carbohydrates (Table 2.4). This inhibitory effect does not appear to be due to osmotic or pH changes. The molar concentration of mannitol which showed no inhibition was equal to that of other carbohydrates showing substantial inhibition of 3-methylindole production. Results obtained with citrate (Table 2.4) were particularly revealing for it exerted a definite inhibitory action on the conversion of L-tryptophan to 3-methylindole in spite of the fact that there was an increase in the pH during incubation rather than a decrease as is noted with other carbohydrates. Starch lowered the pH of ruminal fluid, but did not cause inhibition of 3-methylindole production (Table 2.3).

It has been proposed that the conversion of L-tryptophan to 3-methylindole occurs in two steps involving conversion of L-tryptophan to indoleacetic acid followed by the subsequent decarboxylation of indoleacetic acid to 3-methylindole and that glucose acts by preventing the conversion of indoleacetic acid to 3-methylindole (Yokoyama and Carlson, 1974). In the present study glucose inhibited the conversion

of both L-tryptophan and indoleacetic acid to 3-methylindole (Tables 2.3 and 2.5). Results of studies with carbohydrates *in vitro* should be interpreted with caution. In a trial *in vivo* oral administration of molasses (0.7 kg/cow) every 12 hours) did not prevent the onset of respiratory distress in cattle after oral administration of L-tryptophan (Hammond, Bray, Cummins, Carlson and Bradley, 1978).

It has been suggested that carbohydrate content of grass is possibly an important factor in modulating 3-methylindole production from L-tryptophan in the rumen of cattle under field conditions. This hypothesis is based on speculation that fertilization regimes of grass inducing fog fever are expected to deplete carbohydrate reserves of this grass (Selman *et al*, 1976). *In vitro* studies indicate that the conversion of L-tryptophan to 3-methylindole is due to bacterial activity. The role of ruminal protozoa is less significant since many antibacterial agents strongly inhibited 3-methylindole production (Figs. 2.8 and 2.9 and Tables 2.6. and 2.7). Results obtained with tetracycline were particularly promising; it exhibited strong inhibition of 3-methylindole production in low concentrations (50 µg tetracycline hydrochloride/ml). Assuming that the rumen comprises 20% of the weight of a mature cow, then a dose of 1 g/100 kg body weight would achieve this concentration in the rumen. Results of *in vivo* trials by other investigators (Hammond, Bray, Cummins, Carlson and Bradley, 1978) have shown that oral administration of chlortetracycline (4 g every 12 hours for one day,then 2.5_g for three days) for four days starting one day before L-tryptophan administration reduced ruminal 3-methylindole production and prevented the onset of respiratory distress

after administration of L-tryptophan. For effective inhibition of 3-methylindole production, *in vivo*, oral antibiotic administration would have to commence before L-tryptophan administration and be continued for several days after administration of an oral dose of L-tryptophan. *In vitro* studies indicate that antibacterial agents require to act on ruminal microorganisms for 6-12 hours before achieving substantial inhibition of 3-methylindole production (Figs. 2.8 and 2.9). *In vivo* studies conducted by Hammond, Carlson and Breeze (1978) indicate that oral administration of monensin (100 mg/cow, twice daily for five days, starting 24 hours before L-tryptophan administration) reduced ruminal 3-methylindole production and prevented the onset of respiratory distress after oral administration of L-tryptophan in cattle. Furthermore, monensin reduced 3-methylindole production in the rumen of cattle after abrupt change to lush pasture (Carlson, Hammond, Breeze, Potchoiba and Nocerini, 1981). For effective prevention of the L-tryptophan induced respiratory distress, monensin administration has to be continued after L-tryptophan administration. Oral administration of monensin (200 mg/cow/day) for two weeks ending 48 hours before tryptophan administration did not protect cattle against the L-tryptophan induced pulmonary disease (Hammond, Carlson and Breeze, 1981).

The use of oral administration of antibacterial agents to prevent fog fever under field conditions would be prohibited by the impracticability of repeated oral dosing of grazing cattle. However with poorly soluble compounds a single oral dose could maintain sufficiently high concentrations of the compound for several days.

In this study the poorly soluble sulfonamides, sulfaguanidine and sulfamethoxypyridazine, were evaluated. It was disappointing that none of these compounds caused substantial inhibition of 3-methylindole production at reasonable concentrations. Failure to induce respiratory distress by oral administration of tryptophan prevented the in vivo evaulation of compounds showing strong inhibition of 3-methylindole production, in vitro, in this study.

Yokoyama, Carlson and Holdeman (1977) have isolated a gram positive lactobacillus involved in the conversion of indoleacetic acid to 3-methylindole. However it is not known whether this is an important organism involved in the formation of 3-methylindole and whether it is the only organism involved in the conversion. It is interesting that streptomycin was without effect in inhibiting the conversion suggesting that gram negative organisms are not being involved in the conversion. It might be hypothesized that the conversion is carried out by a number of organisms because the most effective antibiotics were the broad spectrum antibiotics tetracycline and chloramphemicol, while penicillin which is highly active against gram positive species was of lesser effect.

CHAPTER 3

KINETICS OF 3-METHYLINDOLE AND 3-METHYLOXINDOLE IN CATTLE

3.1 - THE METABOLISM OF 3-METHYLINDOLE AND RELATED INDOLIC COMPOUNDS

3.1.1 - The metabolism of certain indolic compounds, related to 3-methylindole in laboratory animals

Excretion of radioactivity from (2-^{14}C) indole when fed to rats was fairly rapid and in two days an average of 81% appeared in the urine, 11% in the faeces and 2.4% as carbon dioxide in the expired air. Radioactivity was excreted in the urine as indoxyl sulphate (50% of the dose), indoxyl glucuronide (11%), oxindole (1.4%, isatin (5.8%), 5-hydroxyindole conjugated (3.1%), N-formylanthranilic acid (0.5%) and unchanged indole (0.07%) (King, Parke and Williams, 1966).

Rats metabolized indoxyl into N-formylanthranilic acid and anthranilic acid, and oxindole into 5-hydroxyoxindole (King et al, 1966; Beckett and Morton, 1966). Thus it has been proposed that the rat metabolizes indole through two routes. A major route is via indoxyl to isatin, N-formylanthranilic acid and anthranilic acid, and the other via oxindole to 5-hydroxyoxyindole and possibly to D-aminophenylacetic acid and anthranilic acid (King et al, 1966).

In vitro studies indicate that rat liver microsomes, under aerobic conditions, metabolize indole to indoxyl, oxindole, possibly isatin, N-formylanthranilic acid and anthranilic acid, but under anaerobic conditions only oxindole is produced (King et al, 1966). Other workers (Beckett and Morton, 1966) reported that incubation of indole with rat liver microsomes yielded only oxindole. Rabbit liver microsomes, on the other hand, did not yield oxindole, but yielded indoxyl (Beckett and Morton, 1966; Posner, Mitoma and Udenfriend,

1961). Oxindole was metabolized to 5-hydroxyoxindole, N-methyloxindole to 5-hydroxy-N-methyloxindole and 3-methyloxindole to 5-hydroxy-3-methyloxindole by rat, guinea pig and rabbit liver microsomes (Beckett and Morton, 1966). These studies indicate that the hydroxylation of indole occurs at position 3 and of oxindole, N-methyloxindole and 3-methyloxindole occurs at position 5 of the indole or oxindole ring (Beckett and Morton, 1966; King et al, 1966) but not at position 6 as was previously reported by King, Parke and Williams (1963).

Ichihara, Sakamoto, Inamori and Sakamoto (1957) reported the 5-hydroxylation of indoles by liver microsomes, but no evidence for this conversion was found in later work by Jepson, Udenfriend and Zaltzmann (1959), Udenfriend, Cleveling, Posner, Redfield, Daly and Witkop (1959) and Posner et al (1961).

Contradictory findings on the metabolism of indoles can be explained because work on these compounds is complicated by their relative chemical instability leading to chemical artefacts.

3.1.2.- Oxidases catalyzing the oxidation of indolic compounds

An enzyme pyrrolooxygenase with the characteristics of mixed function oxidases has been isolated from rat liver microsomes. Pyrrolooxygenase oxidizes the pyrrole moiety of indolic compounds converting them mainly to 2-formamidophenacyl derivatives and oxidizes alkylpyrroles with the formation of substituted pyrrolin-2-ones. The difference in specificity for different substrates allowed classification of the pyrrolooxygenases into skatole pyrrolooxygenase and tryptophan pyrrolooxygenase. Skatole pyrrolo-

oxygenase catalyzes the oxidation of 3-methylindole (skatole) to mainly 2-formamidoacetophenone, and 3-methyloxindole. The 2,3-indole-epoxide has been proposed as the first intermediate in this conversion of 3-methylindole to 3-methyloxindole. However suggestions about the formation of this epoxide are speculative. Authors of this work (Frydman, Tomaro and Frydman, 1972; Frydman, Frydman and Tomaro, 1973) did not attempt to isolate the epoxide because it is highly unstable. They state that this type of epoxide has never been isolated in indole chemistry.

3.1.3 - The pneumotoxic effect and metabolism of 3-methylindole in goats

Hammond, Carlson and Willett (1979) investigated the metabolism of 3-methylindole in goats. Goats were given an infusion into the jugular vein of (^{14}C) 3-methylindole. Most of the radioactivity was excreted in the urine which contained 87 to 92% of the administered radioactivity in three days. Negligible amounts of radioactivity were excreted in expired air (0.4 to 0.9%) and faeces. Composite urine samples were fractionated into ten peaks by ion exchange chromatography which represented 80% of the total urinary radioactivity. This study has shown that a major route of metabolism involved formation of 3-methyloxindole and it was proposed that a mixed function oxidase, pyrrolooxygenase, may be the major metabolic system involved and that a minor route of metabolism involved oxidation of the methyl carbon. Also this study of Hammond, Carlson and Willett (1979) revealed many differences between the metabolism of 3-methylindole by goats and

indole, a closely related compound, by rats. While the major metabolic pathway of indole metabolism in the rat is through aromatic hydroxylation (King et al, 1966), in the case of 3-methylindole, however, the occurrence of the analogous aromatic hydroxylated compounds or their sulphate esters in the urine of goats was not demonstrated after infusion of 3-methylindole. The major metabolic pathway of 3-methylindole was the oxindole route (Hammond, Carlson and Willett, 1979). Another difference was the extent of excretion of conjugated metabolites from 3-methylindole and indole. Less than 20% of the recovered radioactivity in the urine of goats infused with 3-methylindole was in the form of conjugated 3-methylindole metabolites (Hammond, Carlson and Willett, 1979). In the rat indole was excreted mainly as sulphate and glucuronide conjugates (King et al, 1966).

Recent studies suggest that the pneumotoxic effect of 3-methylindole results from the metabolism of 3-methylindole by mixed function oxidases. Bray and Carlson (1979a) have shown that pretreatment with piperonyl butoxide (a mixed function oxidase inhibitor) prevented the onset of acute pulmonary oedema and prolonged the plasma half-life of radioactivity, whereas phenobarbital (a mixed function oxidase inducer) pretreatment caused more severe lesions and a shortened plasma half-life of radioactivity in goats after infusion of radiolabelled 3-methylindole compared to goats receiving 3-methylindole alone. In vitro studies indicate that the metabolism of 3-methylindole by goat microsomal preparations may involve the formation of reactive metabolites which become covalently bound to microsomal proteins (Bray and Carlson, 1979b).

The results of Hammond, Carlson and Willett (1979) show that a major pathway of 3-methylindole metabolism begins with the formation of 3-methyloxindole and a minor pathway begins with the formation of indole-3-carboxylic acid as early metabolites. It has been attempted to determine whether these pathways are involved in the 3-methylindole-induced pneumotoxicity (Potchoiba, Carlson and Breeze, 1981). Administration of 3-methyloxindole or indole-3-carbinol did not cause clinical signs or pathological lesions similar to 3-methylindole-induced pulmonary damage. Urinary metabolites of 3-methyloxindole cochromatographed with 3-methylindole metabolites while urinary metabolites of indole-3-carbinol did not cochromatograph with any major 3-methylindole metabolites. These results confirmed the hypothesis that the oxindole pathway represents a major route of 3-methylindole metabolism whereas the carboxylic acid pathway represents a minor route of metabolism of 3-methylindole in goats and strongly suggests that the 3-methylindole-pneumotoxicity probably results from an early step in the metabolism of 3-methylindole prior to 3-methyl-oxindole or indole-3-carboxylic acid formation (Potchoiba, Carlson and Breeze, 1981).

3.2 - EXPERIMENTAL

3.2.1 - Introduction

Much of the available evidence from other species suggests that the pulmonary toxicity of 3-methylindole in cattle may be due to its metabolic activation to a highly reactive metabolite which alkylates cellular macromolecules causing cellular damage (Chapter 6). However the metabolism of 3-methylindole has not been investigated in cattle although it might be anticipated that the metabolism

of 3-methylindole in cattle would be similar to that in goats reported by Hammond, Carlson and Willett (1979), viz that 3-methylindole is metabolised via 3-methyloxindole to a great extent.

In this series of experiments:

1) 3-Methyloxindole was investigated as a possible metabolite of 3-methylindole in cattle. Its half-life in plasma of calves was measured.

2) The half-life of 3-methylindole and radioactivity in plasma of calves after administration of radiolabelled 3-methylindole were also investigated.

3.2.2 - Materials

t-Butyl alcohol and N-bromosuccinimide were obtained from BDH Chemicals Ltd., Poole, England. The liquid scintillator Unisolve 1 was obtained from Koch-Light Ltd., Colnbrook, Bucks., England.

Tritiated 3-methylindole was prepared as described in Chapter 6. Other chemicals were commercially available and were of analytical grade.

3.2.3 - Preparation of 3-methyloxindole

3-Methyloxindole was prepared according to the method of Hinman and Bauman (1964), viz:

To a solution of 16.4 g of 3-methylindole in 815 ml of 95% t-butyl alcohol was added 22.25 g of N-bromosuccinimide with stirring over a period of 19 minutes. The reaction mixture was kept under nitrogen and at a temperature of 20°C. After 2.5 hours

the solution was concentrated under reduced pressure at room temperature to a volume of a few ml, 30 ml of water was added, and the mixture was extracted with three 125 ml portions of ethyl acetate. The extract was washed with saturated sodium chloride solution, dried over sodium sulphate and evaporated. The residue was recrystallized from water and then twice recrystallized from an acetone-hexane mixture (2:1). Several crops of 3-methyloxindole crystals were collected from the acetone-hexane solvent, giving a total of 3.7 g (23% recovery).

The identity of 3-methyloxindole was confirmed using thin layer chromatography, gas liquid chromatography and by gas liquid chromatography-mass spectrometry.

3.2.4 - Thin layer chromatography of 3-methyloxindole

Plates

Silica gel G (type 60) were prepared as described previously (Chapter 2).

Solvent systems

Solvent 1: Di-isopropyl ether

Solvent 2: Chloroform-ethyl alcohol (19:1)

Solvent 3: Chloroform-acetone (5:4).

These three solvent systems have been used for thin layer chromatography of indoles (Haecock and Mahon, 1963a; Beckett and Morton, 1966).

Detection

Ehrlich's reagent was used for detection of indolic spots and was prepared as described in Chapter 2.

Procedure

5-10 µl aliquots of a solution of 3-methyloxindole in ethylacetate (1 mg/ml) were spotted on thin layer plates. The plates were developed for 30 minutes.

Results

3-Methyloxindole reacted very slowly with Erhlich's reagent giving an orange yellow spot after 24 hours. The Rf values of 3-methyloxindole were 0.25, 0.71 and 0.82 in the solvent systems 1, 2 and 3 respectively.

3.2.5 - Gas-liquid chromatographic determination of 3-methyloxindole in bovine plasma

There is no method available in the literature for determination of 3-methyloxindole. In this present work gas-liquid chromatography was adopted for determination of 3-methyloxindole in plasma of cattle.

1 ml of plasma was extracted with 10 ml of ethylacetate in 15 ml test tubes (glass stoppered). The test tubes were shaken (5 minutes) on a rotary mechanical shaker and the phases were allowed to separate for 5 minutes. The plasma was pipetted off and the ethylacetate layer was dried (solvent washed anhydrous sodium sulphate), 5 ml was concentrated under nitrogen at 50°C to 0.5 ml and a 5 µl aliquot was injected into the gas chromatograph. 3-Methyloxindole concentrations were quantified from known standards in ethylacetate.

Instrument

As described previously (Chapter 2) for determination of

3-methylindole. Column temperature was 150°C or 180°C.

Standards

A series of standard solutions were prepared using 3-methyloxindole dissolved in ethylacetate. 5 µl aliquots were injected into the chromatograph. The standard curve was prepared using the height of the peaks obtained.

Results

On gas-liquid chromatography 3-methyloxindole gave a single peak. A typical gas chromatogram for a standard solution of 3-methyloxindole and 3-methylindole is shown in Fig. 3.1. The resolution of 3-methylindole and 3-methyloxindole was good. The recorder response (height of the peaks) was linearly related to the concentration of 3-methyloxindole.

Fig. 3.1 shows a gas chromatogram for 3-methyloxindole recovered from bovine plasma and of two standard solutions of 3-methyloxindole. None of the constituents of bovine plasma interfered with the assay.

Recoveries

Various concentrations of 3-methyloxindole were prepared in bovine plasma. Plasma samples were stored for one week at -20°C. 3-Methyloxindole concentration was determined in different plasma samples and the recovery was calculated.

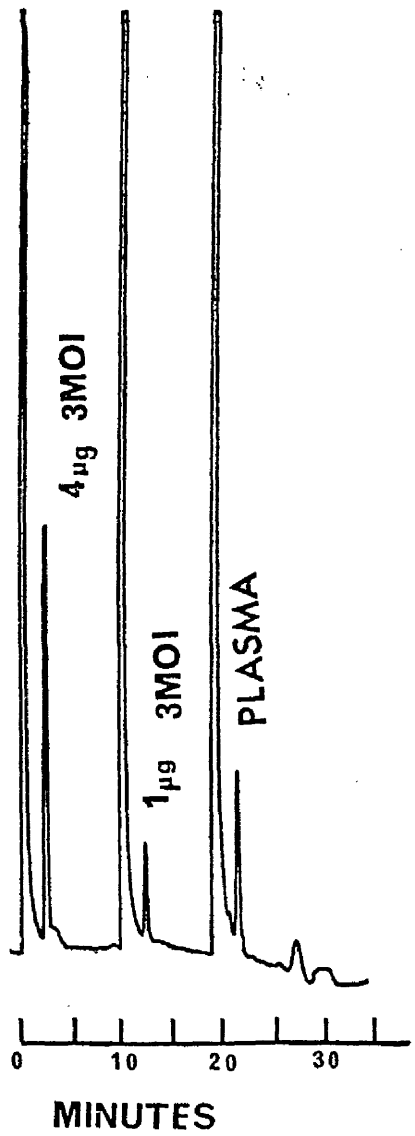
Results

Recoveries of different concentrations of 3-methyloxindole from bovine plasma are shown in Table 3.1.

The ethylacetate extraction procedure used in the present method gave high recovery of 3-methyloxindole (mean ± SEM = 95 ± 3%)

Fig. 3.1 Right panel: A gas chromatogram of a standard solution of a mixture of 3-methylindole (3 MI) and 3-methyloxindole (3MOI) in ethylacetate (gas-chromatograph operated at 150°C column oven temperature).

Left panel: A gas-chromatogram of 3-methyloxindole recovered from plasma (Plasma) and of two standard solutions of 3-methyloxindole in ethylacetate (gas-chromatograph operated at 180°C column oven temperature).


4µg 3MOI
1µg 3MOI
PLASMA
0
10
20
30
MINUTES

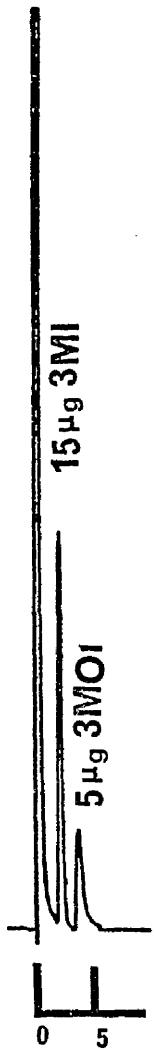

15µg 3MI
5µg 3MOI
0
5
min

TABLE 3.1 RECOVERY OF 3-METHYLOXINDOLE ADDED TO BOVINE PLASMA

3-methyl-oxindole added µg	3-methyl-oxindole measured µg	Mean ± SE	SEM as % of mean	Ratio of assayed to added
5	4.8	4.7 ± 0.1	2	0.94
5	4.6			
10	10.2	9.8 ± 0.4	4	0.99
10	9.5			
20	18.5	18.1 ± 0.5	3	0.91
20	17.6			
40	38.1	37.8 ± 0.3	1	0.95
40	37.5			
80	77.2	76.2 ± 1.1	1	0.95
80	75.1			

3.2.6 - Gas-liquid chromatography-mass spectrometry of 3-methyloxindole

Gas-liquid chromatography-mass spectrometry was performed on a standard solution of 3-methyloxindole in diethyl ether (1 mg/ml).

Results

Results of gas-liquid chromatography-mass spectrometric examination confirmed the identity of 3-methyloxindole. The base peak corresponded to molecular ion peak (parent peak) and had an m/e ratio of 147 (3-methyloxindole = C_9H_9ON = 147). A mass spectrogram of 3-methyloxindole is shown in Fig. 3.2.

3.2.7 - Concentrations of 3-methylindole and 3-methyloxindole in plasma after administration of 3-methylindole in cattle

This experiment was conducted in collaboration with the Department of Veterinary Medicine.

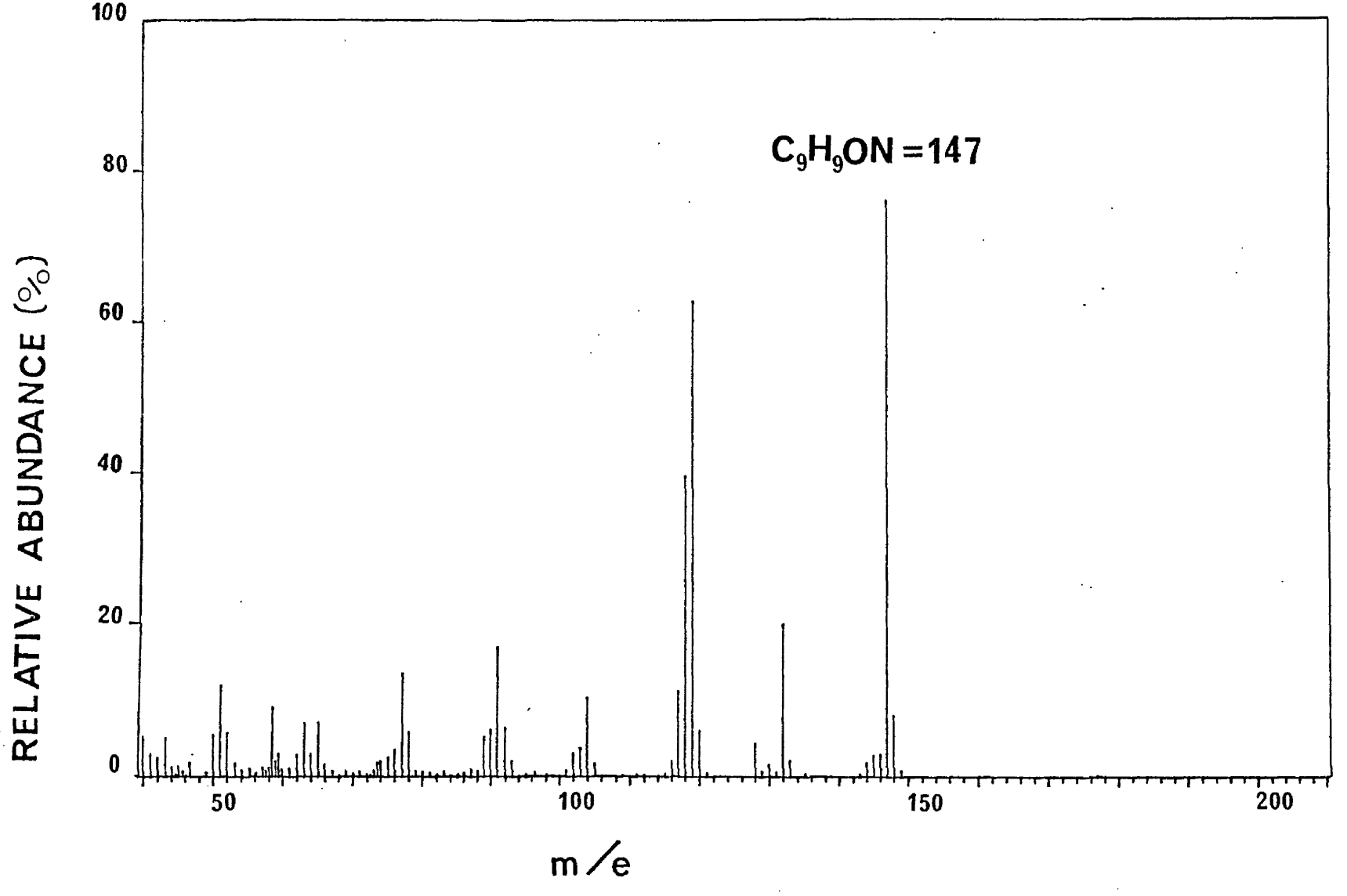
Cattle used in this experiment were adult Hereford and Hereford cross breed females, 500-600 kg body weight, designated A, B, C, D and E. Cattle A, B, C and D each had a permanent tracheal fistula.

Cattle were housed in an open court five days before dosing and during the experimental period. Hay and water were freely offered to all cattle during the experimental period.

3-Methylindole (0.2 g/kg body weight) was administered through a stomach tube as a slurry in approximately 5 litres of water, to animals A, B, C and D; the control animal E received 5 litres of water by the same route.

Clinical examinations were carried out several times daily over this period. Respiratory rates were obtained during the daily clinical examination which was made at 10 a.m. from the day prior to

Fig. 3.2 A mass spectrogram of 3-methyloxindole


100
80
60
40
20
0
RELATIVE ABUNDANCE (%)
C_9H_9ON =147
50
100
150
200
m/e

dosing. Heparinized blood samples were collected at hourly intervals for the first seven hours, then at 24 and 48 hours after 3-methylindole dosing. Plasma was separated and stored at -20°C until analysis within seven days.

3-Methylindole and 3-methyloxindole were determined in plasma, as described previously, using gas-liquid chromatography.

The aliquots of the ethylacetate extract of plasma remaining after determination of 3-methyloxindole were pooled. 10 ml were evaporated to 0.5 ml under nitrogen, then dried (anhydrous sodium sulphate) and filtered. Aliquots of 15 µl were spotted on silica gel G thin layer plates and cochromatographed with 3-methylindole and 3-methyloxindole standards as described before. The ethylacetate extract was also subjected to gas-chromatography-mass spectrometric examination.

Results

3-Methylindole and 3-methyloxindole were identified in plasma of 3-methylindole dosed cows. Thin layer chromatoplates of the combined ethylacetate of plasma samples of these cows showed two spots with Rf values and colour reactions identical with those of authentic samples of 3-methylindole and 3-methyloxindole.

Gas-liquid chromatography of ethylacetate extract of plasma samples showed two peaks with retention times identical to authentic samples of 3-methylindole and 3-methyloxindole.

The identity of 3-methyloxindole recovered from bovine plasma was confirmed by gas-liquid chromatography-mass spectrometry, and gave a mass spectrum identical with that of authentic samples of

3-methyloxindole.

Concentrations of 3-methylindole and 3-methyloxindole in plasma, after administration of 3-methylindole in four cows are shown in Fig. 3.3. Neither compound was n/t detected in plasma of the control cow dosed with water alone. Maximal concentrations of 3-methylindole in plasma were reached eight hours after administration of 3-methylindole to the four cows, whereas the time for maximal concentration of 3-methyloxindole in plasma varied in different cows.

The main clinical findings in cows dosed with 3-methylindole are set out in Table 3.2. In the first 12 hours after dosing each cow given 3-methylindole demonstrated to varying degrees signs of dullness, reduced appetite, trembling, excessive salivation and tachypnoea. Cows A and B developed tachypnoea and hyperpnoea in the 12-96 hours period post dosing, but always remained alert and retained a mild interest in food. Respiratory abnormalities in these animals became less marked over the next few days (96-168 hours) and by the eighth day after dosing tachypnoea was no longer detected in any of them. The cattle were considered to be clinically normal at this time. The other two cattle C and D, however, developed tachypnoea and hyperpnoea in the first 24 hours after dosing and proceeded to deteriorate with respiratory distress becoming more marked 48-60 hours after injection. Cow C died 84 hours after dosing and D died 72 hours after dosing.

Respiratory rates of 3-methylindole dosed and control cattle are shown in Table 3.3.

Fig. 3.3 Plasma 3-methylindole (3MI) and 3-methyloxindole (3MOI) concentrations after oral administration of 3-methylindole (0.2 g/kg) to four cows

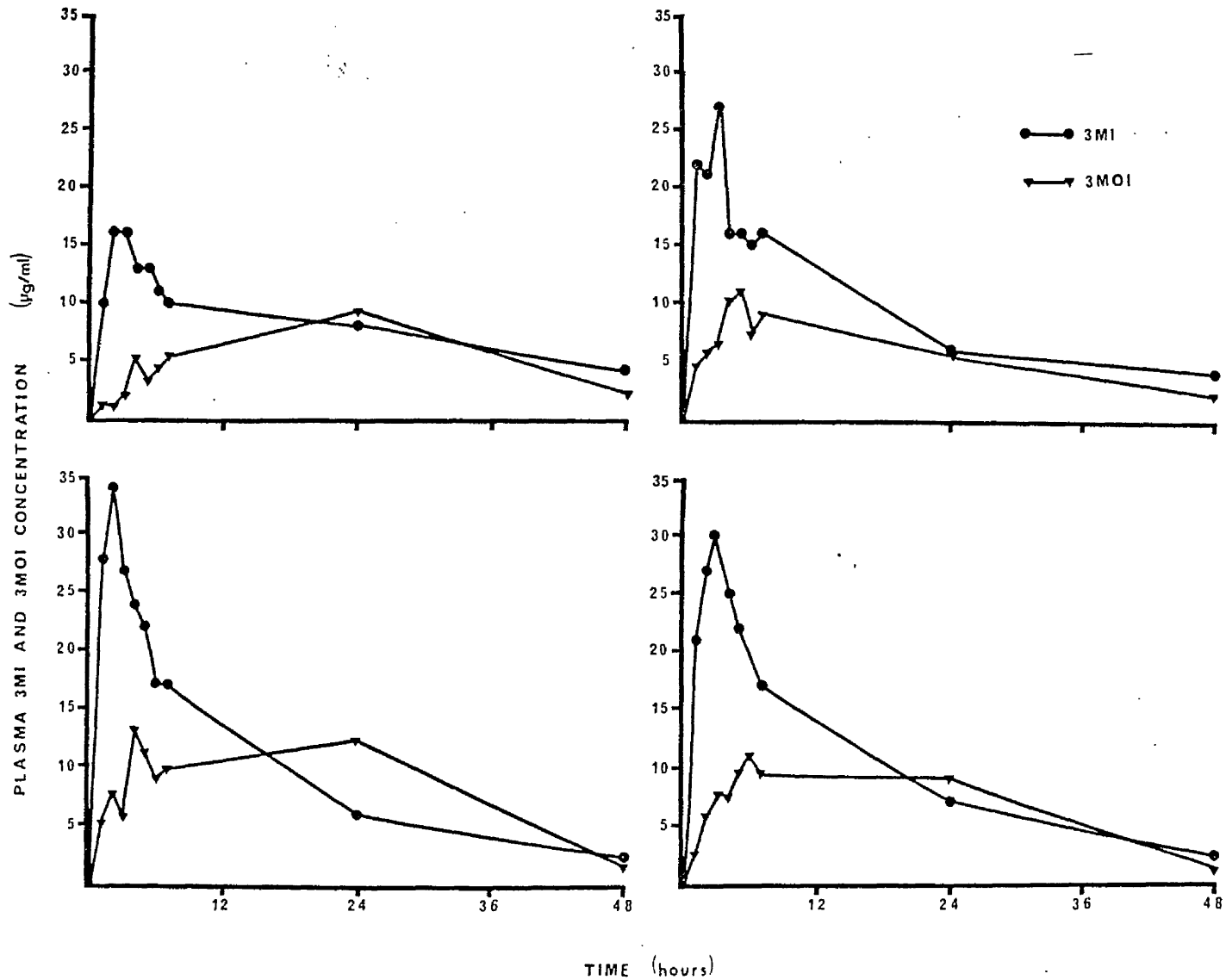

PLASMA 3MI AND 3MOI CONCENTRATION (μg/ml)
35
30
25
20
15
10
5
12
24
36
48
TIME (hours)
3MI
3MOI

TABLE 3.2 MAIN CLINICAL FINDINGS OF FOUR COWS (A,B, C, D) AFTER ORAL ADMINISTRATION OF 3-METHYLINDOLE (0.2 g/kg) AND OF A CONTROL COW (E) GIVEN WATER ALONE

Cow	0-24 hours	24-48 hours	48-96 hours	96-168 hours
A	Dull, marked trembling, reduced appetite, excessive salivation, slight tachypnoea	Dull, but alert, reduced appetite, increasing tachypnoea moderate hyperpnoea	Alert, increasing appetite, decreasing tachypnoea	Bright, good appetite, tachypnoea reduced
B	As above	As above	As above	As above
C	Dull, marked trembling, anorexic, excessive salivation, severe tachypnoea, moderate hyperpnoea	Very dull, anorexic, tachypnoea marked hyperpnoea	Very dull, anorexic, increasing tachypnoea marked hyperpnoea, respiratory distress, death at 84 hours	
D	As above, but tachypnoea	As above, tachypnoea	As above, severe respiratory distress, death at 72 hours	
E	No abnormality detected	No abnormality detected	No abnormality detected	No abnormality detected

TABLE 3.3 RESPIRATORY RATES OF FOUR COWS (A, B, C, D) AFTER ORAL ADMINISTRATION OF 3-METHYLINDOLE (0.2 g/kg) AND OF A CONTROL COW (E) GIVEN WATER ALONE

Time (hours)	Respiratory Rate				
	3-methylindole dosed				Control
	A	B	C	D	E
0	20	20	20	20	20
12	40	40	50	60	20
24	40	40	70	80	20
36	50	50	70	80	20
48	60	60	70	100	20
60	60	50	90	100	20
72	60	50	100	Death	20
84	50	40	Death		20
96	50	40			20
108	40	30			20
120	40	30			20
144	30	20			20
168	30	20			20

3.2.8 - Kinetics of 3-methyloxindole in calves

Three Ayrshire breed calves (42, 50 and 56 kg body weight, designated 1, 2 and 3 respectively) were used.

Animals were anaesthetized with pentobarbitone (20 mg/kg body weight, given intravenously). 3-Methyloxindole was dissolved in 10% Cremophor-EL in distilled water at a concentration of 20 mg/ml. Infusions of 3-methyloxindole were made into the femoral vein via an indwelling cannula and blood sampling for 3-methyloxindole determination was made from the other femoral vein. Each calf was given a dose of 10 mg/kg body weight of 3-methyloxindole. Heparinized blood samples were collected at intervals after 3-methyloxindole injection. Plasma was separated and stored at -20^{o}C until analysed within seven days. 3-Methyloxindole determination was made using gas-liquid chromatography as described before.

Results

The plasma concentrations of 3-methyloxindole are shown in Fig. 3.4. The plasma half-lives of 3-methyloxindole were 10.2, 11.6 and 11.6 in calves 1, 2 and 3 respectively (mean ± SEM = 11.1 ± 0.7 minutes.

3.2.9 - Kinetics of tritiated 3-methylindole in calves

Two Ayrshire breed calves (designated 1 and 2, 36 and 50 kg body weight respectively) were anaesthetized with pentobarbitone (20 mg/kg, intravenously) with additional 2 mg/kg doses given as required throughout the course of the experiment to maintain anaesthesia.

Infusions were made into the femoral vein via an indwelling cannula and blood sampling was from the other femoral vein. (G-^{3}H)

3-methylindole of specific activity 0.15 μCi/mg was dissolved in 10% Cremophor-EL in distilled water (30 mg/ml). Each dose was infused over one minute, with the subsequent dose given after 30 minutes. Blood samples were taken using heparinized syringes at 2, 5, 10, 20 and 30 minutes after each infusion. Plasma was separated and aliquots were taken for determination of total radioactivity and 3-methylindole concentrations.

Total radioactivity in plasma was measured by mixing 0.5 ml plasma with 10 ml of the liquid scintillation cocktail Unisolve 1 in a liquid scintillation vial and radioactivity was determined using a Packard-Tricarb liquid scintillation spectrometer Model 3255. All values were corrected for background radioactivity (less than 10 counts per minute) and quenching (internal standardization method).

Determination of 3-methylindole in plasma was done using gas-liquid chromatography as described before, and 3-methylindole concentrations were converted into equivalent radioactivity.

Results

Radioactivity accounted for as 3-methylindole and total radioactivity concentrations in plasma after successive increasing doses of tritiated 3-methylindole are shown in Figs. 3.5 and 3.6.

Plasma half-lives of 3-methylindole in calves 1 and 2 were 15 ± 3 minutes and 17 ± 5 minutes respectively (mean = 16 minutes).

Plasma half-lives of total radioactivity were 43 ± 15 minutes and 52 ± 15 minutes in calves 1 and 2 respectively (mean = 48 minutes).

Fig. 3.4 Plasma concentrations of 3-methyloxindole (3MOI) after intravenous injection of a dose of 10 mg 3-methyloxindole/kg in three calves

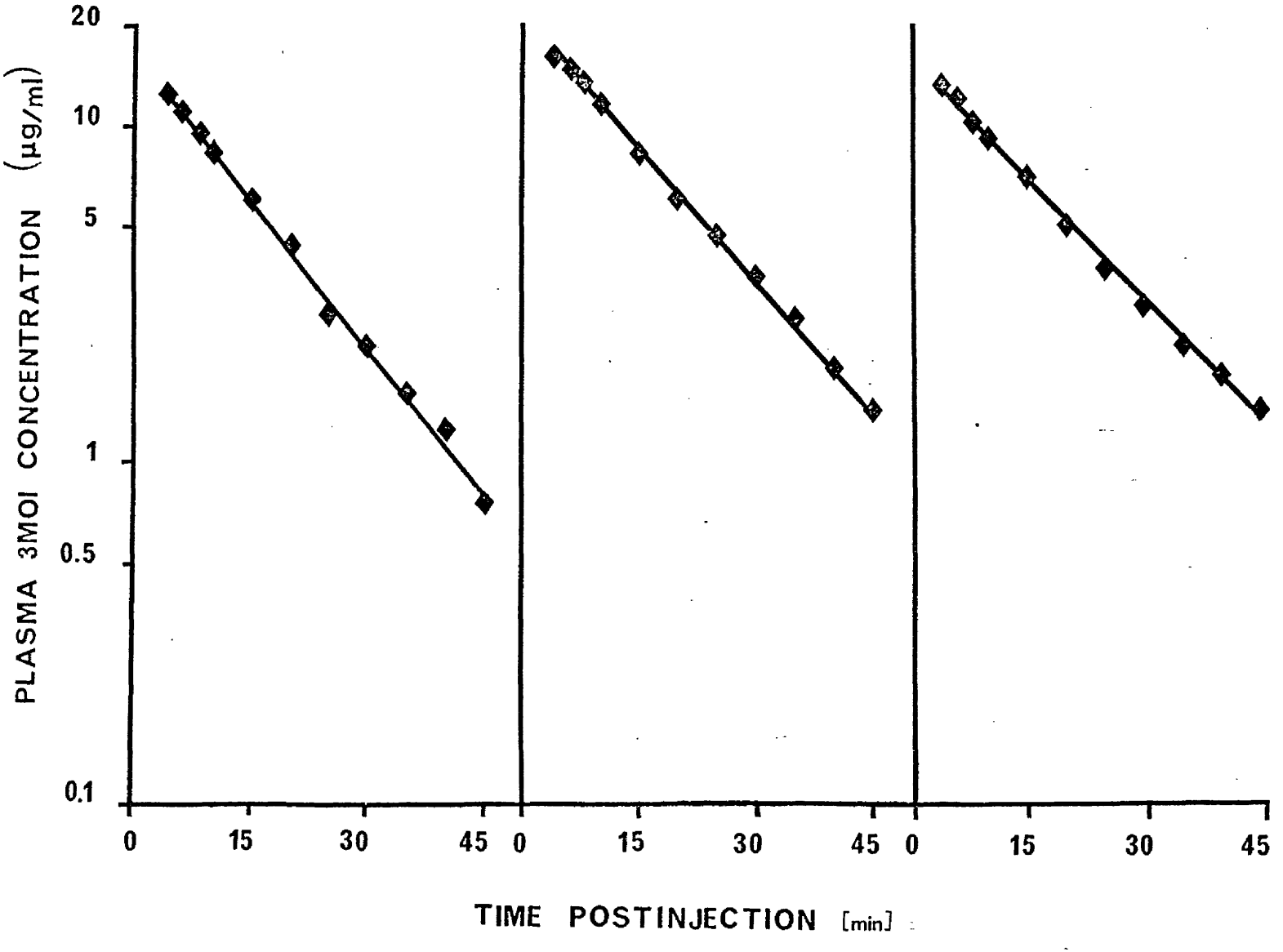

PLASMA 3MOI CONCENTRATION (µg/ml)
20
10
5
1
0.5
0.1
0
15
30
45
0
15
30
45
0
15
30
45
TIME POSTINJECTION [min]

Fig. 3.5 Radioactivity accounted as 3-methylindole (3MI) and total radioactivity (Total) in plasma of Calf 1 after repeated intravenous infusions of tritiated 3-methylindole. Arrows mark the time of injection. A, B, C, D and E were 1, 1, 2, 4 and 12 mg/kg intravenous doses of 3-methylindole, respectively.

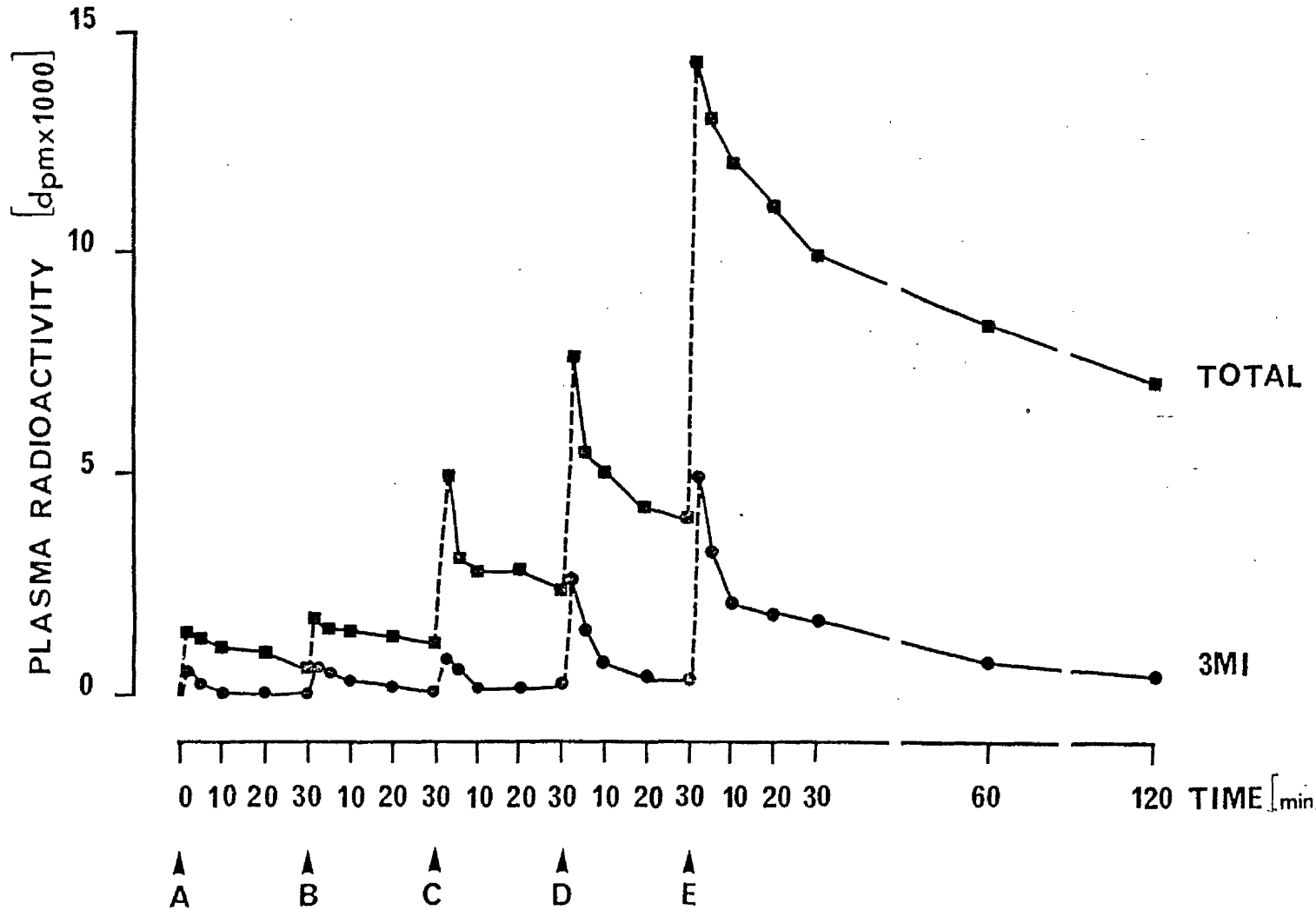
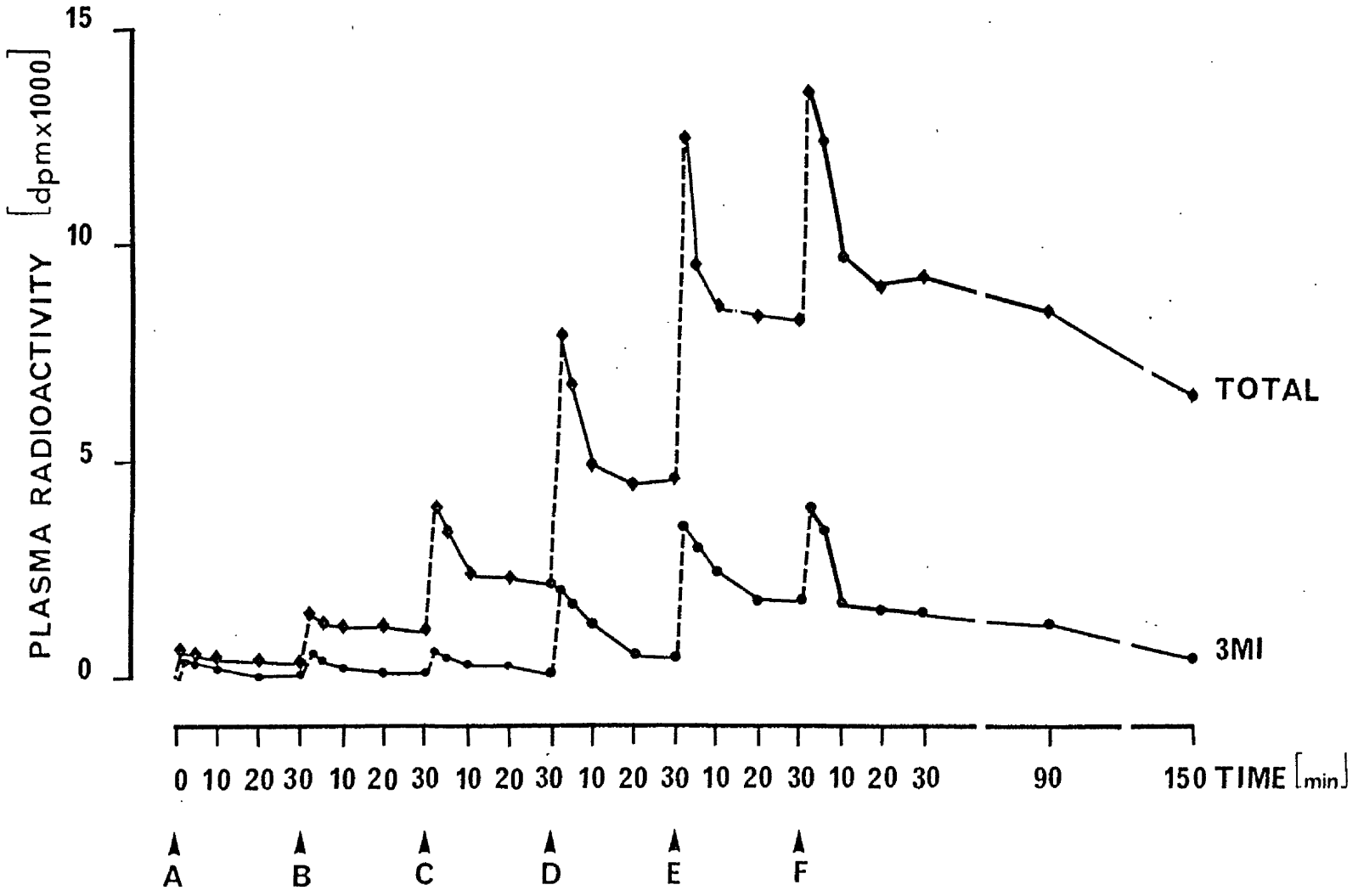

PLASMA RADIOACTIVITY [dpmx1000]
15
10
5
0
TOTAL
3MI
0 10 20 30 10 20 30 10 20 30 10 20 30 10 20 30 60 120 TIME [min]
A
B
C
D
E

Fig. 3.6 Radioactivity accounted as 3-methylindole (3MI) and total radioactivity (Total) in plasma of Calf 2 after repeated intravenous infusions of tritiated 3-methylindole. Arrows mark the time of injection. A, B, C, D, E and F were 1, 1, 2, 4, 8 and 4 mg/kg intravenous doses of 3-methylindole, respectively.


PLASMA RADIOACTIVITY [dpm x 1000]
15
10
5
0
TOTAL
3MI
0 10 20 30 10 20 30 10 20 30 10 20 30 10 20 30 10 20 30 90 150 TIME [min]
A
B
C
D
E
F

3.3 - DISCUSSION

Results of the present work show that the absorption of 3-methylindole is rapid after oral administration in cattle. Maximal concentrations of 3-methylindole in plasma were achieved 2-3 hours after oral administration of a dose of 0.2 g/kg of 3-methylindole in four cattle (Fig. 3.3). Rapid absorption of 3-methylindole is probably due to its high lipid solubility. An earlier investigation (Carlson, Dickinson, Yokoyama and Bradley, 1975) of 3-methylindole absorption was not satisfactory in that the first blood sample was not taken until three hours after 3-methylindole administration was completed which, from these results, is after the time at which 3-methylindole concentrations in plasma are maximal.

The plasma half-lives of 3-methylindole and 3-methyloxindole were remarkably short (16 and 11 minutes respectively) in cattle. Elimination of metabolites of 3-methylindole in cattle is rapid as shown by the rapid decline of radioactivity in plasma after intravenous administration of tritiated 3-methylindole (plasma half-life = 48 minutes).

Because absorption and elimination of 3-methylindole is rapid relative to the rate of production by ruminal microorganisms, it is clear that measurement of either ruminal fluid or plasma concentrations of 3-methylindole in single samples taken under field conditions would not provide any indication of either the rate or extent of 3-methylindole production. Changes in the concentration of 3-methyloxindole in plasma generally paralleled changes in 3-methylindole concentrations. Maintenance of the concentration of 3-methyloxindole

detected in this study (Fig. 3.3) in plasma, of 3-methylindole dosed cattle, would require the conversion of substantial amounts of 3-methylindole to 3-methyloxindole, since the plasma half-life of 3-methyloxindole is short (11 minutes). Therefore this data suggests that 3-methyloxindole is a major metabolite of 3-methylindole in cattle as it is in goats (Hammond, Carlson and Willett, 1979).

Studies (King et al, 1966) on the metabolism of indole indicate that the rat metabolizes indole via two routes, a major route beginning with indoxyl and a minor route beginning with the formation of oxindole. With 3-methylindole, however, Hammond, Carlson and Willett (1979) suggested that the indoxyl route would be blocked by the methyl group and the oxindole route of degradation prevails.

Pyrrolooxygenase, an enzyme system with the characteristics of mixed function oxidases, has been isolated from rat liver microsomes. 3-Methylindole (skatole) pyrrolooxygenase converts 3-methylindole to 3-methyloxindole, 2-formamidoacetophenone and 2-aminoacetophenone. The 2,3-indoleepoxide has been proposed as the first intermediate in the enzymatic oxidation of indolic substrates by pyrroloxygenase (Frydman, Tomaro and Frydman, 1972). This epoxide is highly unstable and cannot be isolated. Isolation and identification of 3-methyl-oxindole in plasma of 3-methylindole dosed cattle would suggest the formation of 3-methyl-2,3-indoleepoxide as an intermediate between 3-methylindole and 3-methyloxindole. The toxological implications of epoxide formation are discussed in Chapter 6.

Clinical observations on 3-methylindole dosed cattle reported in this study and in other studies (Carlson, Dickinson, Yokoyama and

Bradley, 1975) indicate that 3-methylindole dosed cattle continue to deteriorate while the plasma concentration of 3-methylindole was declining, suggesting that 3-methylindole is not acting through a reversible mechanism.

CHAPTER 4

EFFECTS OF 3-METHYLINDOLE AND 3-METHYLINDOLE ANALOGUES ON ISOLATED BOVINE PULMONARY SMOOTH MUSCLES AND AN INVESTIGATION OF 3-METHYLINDOLE AND 3-METHYLOXINDOLE AS POSSIBLE RELEASERS OF MEDIATORS OF ANAPHYLAXIS

4.1 INTRODUCTION

The following approaches can be exploited in the investigation of the mechanism of action of substances which are suspected of releasing substances mediating anaphylaxis in cattle.

4.1.1 - To demonstrate that the substance mimics the effects of anaphylaxis or known releasers of mediator substances in intact cattle

This approach may be complicated by other actions of the administered substances unrelated to the release of mediator substances. For example, compound 48/80 which releases histamine and 5-hydroxy-tryptamine produces an initial rise in blood pressure in calves (Lewis and Eyre, 1972b) which was interpreted as being due to catecholamine release (Lewis and Eyre, 1972b) as reported by Rocha E. Silva (1959). Consistent changes associated with experimentally induced anaphylactic shock induced by challenge with specific antigen in sensitized calves were dyspnoea, coughing, pulmonary hypertension, systemic hypotension and increased salivation, lacrimation and nasal discharges. The pathological changes produced by anaphylaxis after administration of specific antigens to sensitized calves are largely confined to the respiratory system and included intra-alveolar and interstitial oedema and emphysema and intra-alveolar haemorrhage. Petechiae were observed on the epithelial lining of the trachea and on the visceral and parietal pleura. The trachea and major bronchi contained a cream-coloured, frothy fluid which was

often blood-stained (Aitken and Sanford, 1969b; Eyre, Lewis and Wells, 1973).

Sensitized calves developed rapid tolerance to repeated challenge with specific antigen and calves did not respond to a third dose of antigen (Eyre, Lewis and Wells, 1973). Haematological changes in calves undergoing experimentally incuced anaphylaxis were characterized by increased packed cell volume (haemoconcentration), increased potassium concentration (hyperkalaemia) and a severe decrease in neutrophils (neutropenia) (Wells, Eyre and Lumsden, 1973).

Infusion of compound 48/80 to calves caused an initial rise in systemic blood pressure followed by a hypotension leading to death. Pulmonary arterial hypertension and respiratory embarrassment were also recorded. A combination of mepyramine and methysergide protected calves against the effects of compound 48/80 suggesting that the major part of its toxic effect is caused by the release of histamine and 5-hydroxytryptamine. Pathologically, congestion and engorgement of the liver were the only lesions observed in calves after fatal doses of compound 48/80 (Lewis and Eyre, 1972b). The effects of mediators of anaphylaxis on circulatory and respiratory parameters are summarized in Table 4.1.

Symptoms observed after administration of histamine and 5-hydroxytryptamine to conscious calves include: respiratory changes (tachypnoea, dyspnoea and coughing) congestion of the conjunctival mucosa and muzzle and lacrimation. Depression and increased salivary and nasal secretion were marked after histamine, whereas only 5-hydroxytryptamine caused staggering and sneezing. Recovery was

TABLE 4.1 EFFECTS OF MEDIATORS OF ANAPHYLAXIS ON CIRCULATORY AND RESPIRATORY PARAMETERS IN CATTLE

Agonist	Effect on: Systemic arterial blood pressure	Effect on: Pulmonary arterial blood pressure	Effect on: Respiration	Other effects	References
Histamine	Reduction	Reduction	No effect		Burka and Eyre (1974a)
	Reduction	Increase	Apnoea followed by tachypnoea	Decrease in heart rate followed by tachycardia	Aitken and Sanford (1972)
	Reduction	Increase	Large doses caused some inhibition	Small dose: decrease in abdominal vena cava pressure Large dose: decrease followed by rise	Lewis and Eyre (1972b)
5-Hydroxytryptamine	Reduction	Reduction, usually preceded by transient rise	Transient apnoea		Burka and Eyre (1974a)
	Reduction	Increase	Apnoea	Reduction in heart rate	Aitken and Sanford (1972)
	Small dose: decrease Large dose: initial rise followed by rapid fall, then secondary rise and subsequent fall before slow return to normal	Increase	Small dose: no effect Large dose: apnoea	Decrease in abdominal vena cava pressure followed by rise	Lewis and Eyre (1972b)
Bradykinin	Reduction	Reduction			Burka and Eyre (1974a)
	Reduction	Increase	Apnoea	Decrease in heart rate followed by tachycardia	Aitken and Sanford (1972)

TABLE 4.1 (Contd)

Agonist	Effect on				
	Systemic arterial blood pressure	Pulmonary arterial blood pressure	Respiration	Other effects	References
Prostaglandin E_1	Reduction	Reduction	Transient apnoea		Burka and Eyre (1974a)
	Reduction	Increase preceded by transient decrease	Decrease in respiratory volume	Increase in heart rate and increase in abdominal vena cava pressure	Lewis and Eyre (1972a)
	Reduction	Reduction	Decrease in respiratory minute volume	Reduction in heart rate	Aitken and Sanford (1975)
Prostaglandin E_2	Reduction	Reduction	Transient apnoea		Burka and Eyre (1974a)
	Reduction	Increase preceded by transient decrease	Decrease in respiratory volume	Increase in heart rate and increase in abdominal vena cava pressure	Lewis and Eyre (1972a)
	Reduction	Reduction	Decrease in respiratory volume	Decrease in heart rate	Aitken and Sanford (1975)
Prostaglandin $F_{2\alpha}$	Increase	Transient increase	Transient apnoea followed by an increase in respiratory volume		Burka and Eyre (1974a)
	Increase	Increase	Increase in respiratory volume	Increase in heart rate and increase in abdominal vena cava pressure	Lewis and Eyre (1972a)
	Increase	Increase	Decrease in respiratory minute volume	Increase in heart rate	Aitken and Sanford (1975)

observed within less than one hour following administration of non-fatal doses of histamine and 5-hydroxytryptamine (Aitken and Sanford, 1972). Post-mortem examination of calves after administration of histamine revealed severe pulmonary oedema. Less severe oedema was observed in calves which had received bradykinin, whereas 5-hydroxy-tryptamine caused only minimal oedema (Aitken and Sanford, 1972).

Intradermal injection of "bovine slow reacting substance of anaphylaxis" caused dose dependent vascular permeability changes in the skin of calves. These permeability changes caused well defined indurations similar to weals induced by histamine but different from those induced by prostaglandin E_2 (Burka and Eyre, 1977a).

4.1.2 - To demonstrate that the suspected mediator releaser causes changes in blood and tissue concentrations of mediator substances similar to those produced by anaphylaxis or following administration of known chemical releasers of mediator substances

Immunological and chemical release of mediators of anaphylaxis would be expected to increase the concentrations of these mediators in blood and to deplete tissue stores of pre-formed mediators. However this approach is often complicated by considerable individual variation in the concentrations of tissue mediator (e.g. histamine) which tends to mask any changes due to anaphylaxis (Aitken, 1970).

In calves undergoing anaphylaxis, plasma concentration increased during anaphylaxis whereas whole blood histamine concentration fell sharply. This decrease in total blood histamine was attributed

to severe leucopenia accompanying anaphylactic shock (Eyre et al, 1973). Most of the blood histamine is present in formed elements principally the leucocytes of bovines (Holroyde and Eyre, 1975) as in other animal species (Greaves and Mongar, 1968).

Studies on tissue histamine concentration indicate that there is a marked individual variation in tissue histamine in cattle. These individual variations made it impossible to draw any conclusions as to changes in tissue histamine concentration or mast cell morphology in calves undergoing anaphylaxis (Aitken, 1970; Eyre et al, 1973) and following the administration of compound 48/80 (Lewis and Eyre, 1972b). 5-Hydroxytryptamine did not show significant changes during anaphylaxis (Eyre et al, 1973).

Infusion of compound 48/80 in calves caused a significant increase in the concentrations of histamine in whole blood and 5-hydroxytryptamine in plasma (Lewis and Eyre, 1972b).

Production of kinins following bovine anaphylactic shock has been demonstrated. Challenge of sensitized calves with specific antigen resulted in an increase in blood kinin activity. The kinin activity in blood was correlated well with the fall in systemic blood pressure seen in these calves after intravenous injection of the specific antigen (Eyre and Lewis, 1972).

4.1.3 - To demonstrate that antagonists of mediators of anaphylaxis alleviate effects of the suspected mediator releaser

Table 4.2 lists antagonists of known mediators of anaphylaxis in cattle. The effects of these antagonists is often difficult to interpret as many of these antagonists, e.g. sodium meclofenamate, have

TABLE 4.2 ANTAGONISTS OF MEDIATOR OF ANAPHYLAXIS IN CATTLE

Mediator	Antagonist	Experimental Model	References
Histamine	Sodium meclofenamate	Circulatory and respiratory parameters of anaesthetized calves	Burka and Eyre (1974a)
		Bovine cutaneous vasculature (in vivo)	Burka and Eyre (1977b)
	Diethylcarbamazine citrate	Circulatory and respiratory parameters of anaesthetized calves	Burka and Eyre (1974a)
		Isolated bovine pulmonary vein	Eyre (1971c)
	Disodium cromoglycate	As above	As above
	Tripelennamine	As above	As above
	Mepyramine	As above	As above
		Circulatory and respiratory parameters of anaesthetized calves	Aitken and Sanford (1972)
	PR-D-92-EA FPL 55712	Bovine cutaneous vasculature (in vivo)	Burka and Eyre (1977b)
5-Hydroxytryptamine	Diethylcarbamazine citrate	Circulatory and respiratory parameters of anaesthetized calves	Burka and Eyre (1974a)
		Isolated bovine pulmonary vein	Burka and Eyre (1974c)
	Methysergide	As above	Eyre (1971c)
	Disodium cromoglycate	As above	As above

TABLE 4.2 (Contd)

Mediator	Antagonist	Experimental Model	References
Bradykinin	Sodium meclofenamate	Circulatory and respiratory parameters in anaesthetized calves	Aitken and Sanford (1972), and Burka and Eyre (1974a)
Acetylcholine	Sodium meclofenamate	As above	Burka and Eyre (1974a)
Bovine slow reacting substance of anaphylaxis	Sodium meclofenamate	Isolated calf bronchus	Burka and Eyre (1977a)
	PR-D-92-EA	Bovine cutaneous vasculature (in vivo)	Burka and Eyre (1977b)
	FPL 55712	As above	As above
PGE_1	Sodium meclofenamate Indomethacin Phenylbutazone Diethylcarbanazine citrate	Circulatory and respiratory parameters of anaesthetized calves	Burka and Eyre (1974_a)
PGE_2	Sodium meclofenamate	Circulatory and respiratory parameters of anaesthetized calves	Burka and Eyre (1974a)
		Isolated bovine pulmonary vein	Burka and Eyre (1974c)
	Phenylbutazone	Circulatory and respiratory parameters of anaesthetized calves	Burka and Eyre (1974a)
		Isolated bovine pulmonary vein	Burka and Eyre (1974c)
	Diethylcarbamazine citrate	Circulatory and respiratory parameters of anaesthetized calves	Burka and Eyre (1974a)
		Isolated bovine pulmonary vein	Burka and Eyre (1974c)

TABLE 4.2 (Contd)

Mediator	Antagonist	Experimental Model	References
PGE_2	Indomethacin	Circulatory and respiratory parameters of anaesthetized calves	Burka and Eyre (1974a)
	Phloretin phosphates	Isolated bovine pulmonary vein	Burka and Eyre (1974c)
$PGF_{2\alpha}$	Sodium meclofenamate	Circulatory and respiratory parameters of anaesthetized calves	Burka and Eyre (1974a)
	Diethylcarbamazine citrate	Isolated bovine pulmonary vein	Burka and Eyre (1974c)
	Phenylbutazone	As above	As above

a diverse effect and are capable of antagonizing several mediators (Table 4.2).

It has been shown that meclofenamate pre-treatment regularly prevented collapse and respiratory distress in sensitized conscious calves after challenge with specific antigen. Cyproheptadine and methysergide did not modify the anaphylactic response, while atropine and mepyramine reduced lacrimal and other secretions but did not affect collapse and dyspnoea (Aitken and Sanford, 1969a).

Meclofenamate, phenylbutazone, acetylsalicyclic acid and indomethacin protected calves from anaphylactic cardiovascular shock, whereas SC-19220, polyphloretin phosphate and diethylcarbamazine citrate afforded only weak protection against systemic anaphylaxis (Burka and Eyre, 1974a). Based on these findings it has been suggested that prostaglandins are relatively important mediators in cattle (Burka and Eyre, 1974a) since aspirin, indomethacin, phenylbutazone and meclofenamate are all known to be inhibitors of prostaglandin synthesis (Vane, 1971; Flower, Gryglewski, Herbacynska-Cedro and Vane, 1972).

Mepyramine did not inhibit the anaphylactic reaction of sensitized calves to intravenous injection of specific antigen. Methysergide and diethylcarbamazine each suppressed the reaction by about 50%. Disodium cromoglycate alone did not inhibit the anaphylactic response but was synergistic with diethylcarbamazine (Eyre et al, 1973).

Haemoconcentration and hyperkalaemia accompanying anaphylaxis in calves were strongly inhibited by sodium meclofenamate

and by disodium cromoglycate given simultaneously with diethylcarbamazine Sodium meclofenamate strongly inhibited the pathological changes associated with anaphylaxis but disodium cromoglycate and diethyl-carbamazine reduced these changes only slightly. Antihistamine and anti-5-hydroxytryptamine drugs were not effective in preventing haematological or pathological changes associated with anaphylaxis in calves (Wells et al, 1973).

4.1.4 - To demonstrate that the test substances release mediator substances from lung tissue or leucocytes in vitro

Chopped lung tissue and suspensions of leucocytes have been successfully used to demonstrate the release of mediators of anaphylaxis in vitro by immunological and chemical stimuli. Specific antigen and compound 48/80 released dopamine (Eyre, 1971b), histamine (Eyre, 1971a) and 5-hydroxytryptamine (Eyre, 1972) from chopped lung tissue of sensitized calves. In addition specific antigen caused the release of "slow reacting substance of anaphylaxis" from sensitized lung (Burka and Eyre, 1974b).

These results contradict the findings of Aitken (1970) who reported that only tissues from the skin of the muzzle and ear of sensitized calves released histamine into the supernatant fluid when challenged with specific antigen. Tissues from the lung, pleura and liver capsule did not show evidence of release of histamine. 5-Hydroxytryptamine was not detectable in supernatant fluid after incubation of chopped lung tissue from sensitized calves with specific antigen for 60 minutes. Furthermore Aitken (1970) reported that incubation of chopped lung tissue with 5-hydroxytryptamine resulted

in a progressive decrease in the concentration of 5-hydroxytryptamine indicating that it was removed by lung tissue from the incubation fluid.

Incubation of fragments of tissue from the liver capsule, pleura and skin of muzzle and ear with the compound 48/80 caused the release of histamine into the supernatent fluid (Aitken, 1970).

The chopped lung preparation has been shown to be a useful model for evaluation of anti-anaphylactic drugs. The anti-anaphylactic drugs PR-D-92-EA and M & B 22,948 effectively inhibited the immunologic release of the "slow reacting substances of anaphylaxis" from chopped lung preparations. Chlorophenesin and diethylcarbamazine citrate were effective also but only at high concentrations, whereas meclofenamate and aspirin potentiated the release of the "slow reacting substance of anaphylaxis".

The immunological release of histamine from suspensions of polymorphonuclear leucocytes of sensitized calves has been demonstrated (Holroyde and Eyre, 1975).

Attempts to use the isolated perfused bovine lung as an experimental model to investigate the release of mediator substances by immunological stimuli have met with little success. No increase in the concentration of histamine and 5-hydroxytryptamine in the perfusion fluid was observed when isolated perfused lungs from sensitized calves were exposed to specific antigen (Eyre et al, 1973). It has been claimed that challenge of perfused isolated lung preparations with specific antigen resulted in the release of histamine, kinin and other unidentified mediator substances (Aitken, 1970).

4.2 - EXPERIMENTAL

4.2.1 - Introduction

Intravenous injection of 3-methylindole is followed by circulatory and respiratory changes similar to those observed during experimental anaphylaxis in cattle. These changes are characterized by systemic hypotension, pulmonary arterial hypertension and respiratory embarrassment, rapid developrent of tolerance on repeating the initial dose of 3-methylindole and reduction or abolition of the effects of 3-methylindole by antagonists to known mediators of anaphylaxis. Based on these observations it has been suggested that 3-methylindole is acting through release of mediators of anaphylaxis (Atkinson, Bogan, Breeze and Selman, 1977).

Another hypothesis (Eyre, 1975) on the mechanism of the pathogenesis of the L-tryptophan induced pulmonary disease suggests that 3-methylindole and other tryptamine analogues absorbed from the rumen are acting directly on the bovine pulmonary vein causing a primary hydrodynamic imbalance favouring transudation over reabsorption resulting in pulmonary oedema. This hypothesis is based on observations that tryptamine analogues contract the pulmonary vein. The concentrations of 3-methylindole used in this study (Eyre, 1975) were considerably higher than those reported as being present in the plasma of cattle receiving toxic oral doses of 3-methylindole.

In the following experiments the effects of 3-methylindole and 3-methylindole analogues including 3-methyloxindole on isolated smooth muscle preparations from the vasculature and airways of the bovine lung were investigated. Also the possibility that 3-methylindole

or 3-methyloxindole are causing release of mediator substances was investigated using chopped lung preparations.

4.2.2 - Materials

5-Methylindole and 7-methylindole were obtained from Aldrich Chemical Company Inc., Gillingham, Dorset, England.

3-Phenylindole was obtained from Organisch Chem. Inst. T.N.O., Utrecht, Holland.

5-Hydroxytryptamine-creatinine sulphate, histamine dihydrochloride, isoamylalcohol, compound 48/80 and O-phthaldialdehyde were obtained from Sigma Chemical Co. Ltd., London.

Prostaglandin $F_{2\alpha}$ tromethamine was obtained from Upjohn Limitec, Agricultural Veterinary Division, Fleming Way, Crawley, Sussex, England.

3-Methyloxindole was prepared as described in Chapter 3.

Other chemicals were obtained from commercial sources as described previously and were of analytical grade.

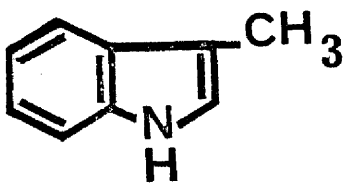
The chemical structures of 3-methylindole and 3-methylindole analogues are illustrated in Fig. 4.1.

4.2.3 - Effects of 3-methylindole analogues on isolated bovine pulmonary smooth muscle preparations

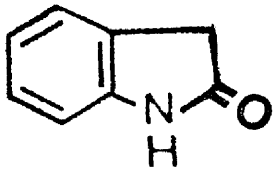
Isolated smooth muscle preparations were obtained from six calves (Ayrshire , 3-12 months old). Both lungs attached to the base of the heart were obtained immediately after slaughter, placed in cold Krebs - Henseleit solution and transported to the laboratory. The isolated bovine pulmonary vein was prepared as described by Aitken (1970). The principal pulmonary veins of both lungs were carefully

Fig. 4.1 Chemical structures of 3-methylindole and 3-methylindole analogues

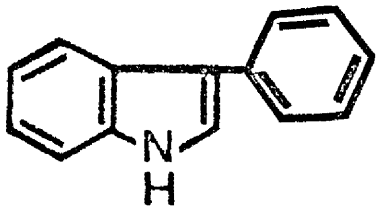
3-methylindole



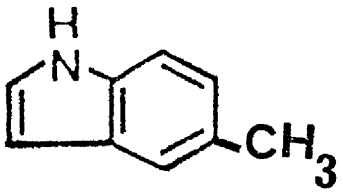
3-methyloxindole



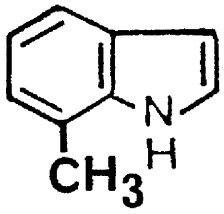
3-phenylindole



5-methylindole



7-methylindole



dissected, removed and placed in Krebs-Henseleit solution. The vein was cut into a single strip, approximately 3-5 mm width and 3-5 cm length, and set up in a 75 ml isolated organ bath at 37°C in Krebs-Henseleit solution aereated with 5% carbon dioxide in oxygen, at a resting tension of about 2 g. The principal pulmonary artery was similarly prepared and set up.

The tracheal muscle was set up as described by Offermeier and Ariëns (1966). A tracheal ring was dissected from the trachea. The tracheal muscle was carefully dissected from the inside of the ring, and set up in an isolated organ bath as described for the pulmonary vein. A bronchus (approx. 5 mm diameter) was carefully dissected, cut spirally into a single strip (4 cm long) (Aitken, 1970; Burka and Eyre, 1977a) and set up as described for the pulmonary vein. Contractions of isolated smooth muscle preparations were recorded isotonically using a linear motion transducer and pen recorder.

Tissues were allowed to equilibrate for 45 minutes before application of drugs. Doses of 3-methylindole analogues (5, 10, 20, 40, 80, 160, 320 and 640 µg/ml) were allowed to act for 15 minutes. Doses of 5-hydroxytryptamine were allowed to act for 5 minutes or for 15 minutes for the bronchus which needed a longer time to contract, or until maximal contractions were reached. 5-Hydroxytryptamine-creatinine sulphate was dissolved in Krebs-Henseleit solution.

3-Methylindole, 3-methyloxindole, 3-phenylindole, 5-methylindole and 7-methylindole were dissolved in 10% "Cremophor EL" in Krebs-Henseleit solution.

The effects of each compound were investigated on isolated

pulmonary vein, pulmonary artery, tracheal muscle and bronchial strip from each of the six calves.

Results

Doses of 3-methylindole, 3-methyloxindole, 3-phenylindole, 5-methylindole and 7-methylindole of 5-640 µg/ml did not cause contraction or relaxation in any of the isolated smooth muscle preparations. 5-Hydroxytryptamine-creatinine sulphate caused dose dependent contraction of the tracheal muscle (0.04-5.12 µg/ml), the pulmonary vein (0.04-1.28 µg/ml). pulmonary artery (0.32-20 µg/ml) and bronchus (0.015-5.12 µg/ml).

4.2.4 - Investigation of 3-methylindole and 3-methyloxindole as possible releasers of mediator substances from chopped bovine lung preparations

A total of six experiments was carried out. The effect of each of 3-methylindole and 3-methyloxindole was assessed on chopped lung preparations from three calves (Ayrshire, 3-12 months old). Lung tissue from one calf was used in each experiment. Chopped bovine lung preparations were prepared according to the technique of Eyre (1971a).

Animals were stunned using a captive bolt pistol, pithed with a light cane, and immediately exsanguinated by jugular section. Lungs were removed as soon as possible and placed in chilled Tyrode solution (4^{o}C). Lungs showing macroscopic abnormalities were discarded. Lung tissue was chopped into pieces approximately 4 mm in diameter with a pair of scissors. The chopped tissue was placed in an excess

of Tyrode solution and left to wash for one hour at 4°C with three changes of Tyrode.

Pieces of tissue were blotted on filter paper, weighed as accurately as possible and 2 g aliquots suspended in 4.5 ml Tyrode solution in conical flasks (25 ml capacity). A total of 15 flasks was prepared in this way and divided into three equal groups for incubation with Tyrode alone, Tyrode containing compound 48/80 and Tyrode containing 3-methylindole or 3-methyloxindole (which were tried in separate experiments). Flasks were incubated in a shaking water bath at 37°C with continuous oxygenation (a mixture of 5% carbon dioxide in oxygen was bubbled through the flasks slowly to avoid excessive frothing in flasks containing "Cremaphor-EL") for 20 minutes. Incubation commenced by adding 0.5 ml of Tyrode alone or Tyrode containing the compound 48/80 (4 mg/ml) or Tyrode containing 3-methylindole (200 µg/ml) or 3-methyloxindole (250 µg/ml) and left to incubate for a further 20 minutes. After incubation, supernatants were removed (Pasteur pipette) and centrifuged (2000 rpm for 20 minutes).

Supernatants and chopped lung tissue of one flask from each group were separated for spectrofluorimetric assay of histamine. Hydrochloric acid was added to these samples immediately to stabilize histamine. 0.5 ml 0.1N HCl was added to 4 ml supernatant and chopped lung tissue (2 g) was diced in 1 ml 0.1N HCl. Acidified tissue and supernatants were stored at -20°C until spectrofluorimetric determination of histamine within seven days. The remaining chopped tissue was discarded and supernatants of each group pooled for bioassay. Bioassay of supernatants on the isolated guinea pig ileum

was carried out on the same day and on the isolated rat uterus in oestrus next day and were stored overnight at -20°C.

4.2.5 - Investigations on mediator substances released from chopped lung tissue

Mediator substances in supernatants were examined by studying the effect of supernatants of chopped lung preparations on the isolated guinea pig ileum which is sensitive to histamine (Vugman and Rocha E. Silva, 1966) and "slow reacting substance" (Chakravarty, 1959) and on the isolated rat uterus which is sensitive to 5-hydroxytryptamine (Offormeier and Ariens, 1966) and prostaglandins (Orange and Austen, 1969). Histamine release was determined using a spectrofluorimetric technique.

4.2.6 - Action of supernatants of chopped lung preparations on the isolated guinea pig ileum

A guinea pig (200 g approximately) was killed with a blow on the head and bled from the jugular vein. The abdomen was opened through a midline incision, the abdominal contents exposed and the ileoceacal junction identified. The terminal portion of the ileum was taken out (about 10 cm) and its lumen carefully washed with warm Tyrode solution. After washing, a small piece of the gut (2-3 cm) was cut. Both ends of the piece were cut obliquely so that the cut ends could be tied while leaving both ends open, allowing perfusion fluid to flow freely in and out of the gut. One end was tied to the bottom of the organ bath while the other end was tied to the lever of a linear motion transducer. Tyrode solution was used as the perfusion fluid at

37°C in a 10 ml organ bath with an overflow connection. Oxygen was continuously bubbled through the bath throughout the experiment.

Doses of equal volumes of supernatants of chopped lung preparations were applied in a random fashion (Latin square design) and the height of the contractions caused by these supernatants were measured. Atropine sulphate (0.1 µg/ml) and mepyramine maleate (1 µg/ml) were used as antagonists to identify mediators.

The height of contractions of the guinea pig ileum produced by different supernatants was compared using analysis of variance.

The height of contractions produced by supernatants of chopped lung containing 3-methylindole, 3-methyloxindole or compound 48/80 was each compared with those produced by supernatants of control chopped lung incubated in Tyrode alone using a paired Student t-test.

The effect of 3-methylindole (2 µg/ml) and 3-methyloxindole (2.5 µg/ml) on the response of the isolated guinea pig ileum to histamine was examined by comparing (paired Student t-test) the height of contractions produced by equal volumes of two standard solutions, one of histamine (200 ng/ml) alone in Tyrode solution and the other of histamine (200 ng/ml) in Tyrode solution containing 3-methylindole (40 µg/ml) or 3-methyloxindole (50 µg/ml).

Results

Supernatants of chopped lung preparations caused contraction of the isolated guinea pig ileum. The height of contractions caused by supernatants of chopped lung preparations incubated in Tyrode solution alone or with compound 48/80, 3-methylindole or 3-methyloxindole is shown in Table 4.3. Analysis of variance showed a significant

TABLE 4.3 RESPONSES OF THE GUINEA PIG ILEUM (CONTRACTIONS, DIVISIONS ON THE RECORDER SCALE) TO SUPERNATANTS OF CHOPPED LUNG PREPARATIONS FROM SIX CALVES

Supernatant	Calf No.																	
	I			II			III			IV			V			VI		
A	5	3	5	13	10	12	12	15	13	8	12	11	9	8	9	10	11	10
B	4	3	5	13	8	10	13	14	15	10	9	8	7	8	6	11	11	12
C	12	10	13	25	20	19	52	55	50	23	21	24	19	20	21	16	18	16

A: 0.5 ml of supernatant of control chopped lung incubated in Tyrode solution alone.

B: 0.5 ml of supernatant of chopped lung incubated in Tyrode solution containing 3-methylindole (Calves I, II and III) or Tyrode solution containing 3-methyloxindole (Calves IV, V and VI).

C: 0.5 ml of supernatant of chopped lung incubated in Tyrode solution containing compound 48/80.

difference ($P < 0.01$) between the height of contractions produced by different supernatants. Closer analysis using paired Student t-test showed no significant difference between the height of contractions produced by supernatants of chopped lung preparations incubated in Tryode solution alone and those produced by supernatants of chopped lung preparations incubated in Tyrode solution containing 3-methylindole or 3-methyloxindole, but supernatants of chopped lung preparations incubated in compound 48/80 produced significantly higher ($P < 0.05$) contractions than those produced by supernatants of chopped lung preparations incubated in Tyrode solution alone.

Supernatants of chopped lung preparations incubated in Tyrode solution alone or in Tyrode solution containing 3-methylindole or 3-methyloxindole did not cause contraction of the guinea pig ileum in the presence of a mixture of atropine sulphate (0.1 µg/ml) and mepyramine maleate (1 µg/ml). Also mepyramine maleate (1 µg/ml) alone blocked these contractions. This mixture of antagonists decreased the height, but did not block completely contractions caused by supernatants of compound 48/80 incubated chopped lung preparations.

The presence of 3-methylindole (2 µg/ml) and 3-methyloxindole (2.5 µg/ml) did not alter the response of the guinea pig ileum to histamine.

4.2.7 - Action of supernatant of chopped lung preparations on the isolated oestrus rat uterus

Rats were pre-treated with oestrogen (oestradiol benzoate, 0.1 mg/kg, s.c., in 0.1 ml ethanol) 18 hours before the bioassay. Rats were killed with a blow on the head and bled from the jugular vein.

The abdomen was opened through a mid line incision and the uterus dissected out and suspended in a 10 ml organ bath. The perfusion fluid used was de Jalon's solution, gassed with 5% carbon dioxide in oxygen at 32°C. Only preparations showing no spontaneous activity under the bioassay conditions described were used. Different doses of supernatants of 3-methylindole, 3-methyloxindole and Tyrode incubated chopped lung and two standard solutions of 5-hydroxytryptamine-creatinine sulphate and prostaglandin $F_{2\alpha}$ tromethamine were allowed to act for two minutes.

Prostaglandins in the supernatants were concentrated by extraction with ethyl acetate and resuspension in a smaller volume of de Jalon solution. Supernatants of 3-methylindole, 3-methyloxindole and Tyrode incubated chopped lung preparations (10 ml) were acidified with 5.75N HCl (0.5 ml) and immediately extracted twice with an equal volume of ethyl acetate. The combined ethyl acetate phases were evaporated to dryness under nitrogen at 50°C (Gilmore, Vane and Wyllie, 1968). The residue was suspended in de Jalon solution (2 ml) before bioassay on the oestrus rat uterus. Doses of the ethyl acetate extract and a standard solution of prostaglandin $F_{2\alpha}$ tromethamine were allowed to act for two minutes.

Results

Doses of 0.5 and 1 ml of supernatants of 3-methylindole, 3-methyloxindole and Tyrode solution incubated chopped lung preparations and of ethyl acetate extracts of supernatants did not cause contraction or relaxation of the oestrus rat uterus. 5-Hydroxytryptamine (10 ng/ml) and prostaglandin $F_{2\alpha}$ (5 ng/ml) caused contraction of the oestrus rat uterus.

4.2.8 - Spectrofluorimetric determination of histamine in chopped lung tissue and in supernatants of chopped lung preparations

Histamine in supernatants and chopped lung tissue was determined using the spectrofluorimetric assay of Anton and Sayre (1969). In this method histamine was selectively extracted into isoamyl alcohol, a fluorophor was formed by a reaction between histamine and O-phthaldialdehyde at alkaline pH, and the fluorescence of the fluorophor was maximized in the presence of citric acid.

Reagents

Washed isoamyl alcohol was prepared by washing isoamyl alcohol once with 1/5 volumes of 1N hydrochloric acid, then three times with equal volumes of water and dried over anhydrous sodium sulphate. 2M Citric acid was prepared using distilled water. O-Phaldialdehyde was twice recrystallized from redistilled n-hexane. O-Phthaldialdehyde reagent was prepared by dissolving O-phaldialdehyde in methanol (5 mg/ml).

Extraction

Extraction was carried out in 50 ml glass-stoppered glass test tubes. Centrifugation was carried out at 2000 rpm for five minutes in a refrigerated centrifuge unless otherwise indicated. Lung tissue (2 g) was homogenized in 4 ml of 0.4N perchloric acid in a glass-glass homogenizer submerged in ice. The homogenate was centrifuged at 3000 rpm for 20 minutes. The supernatant was removed as completely as possible, the residue resuspended in perchloric acid (4 ml), shaken vigorously, and the centrifugation repeated. The supernatant fractions of the perchloric acid extract were pooled in a 10 ml

measuring cylinder. Water was added to make a volume of 10 ml. 2 ml were transferred to a 50 ml test tube containing 2 ml water.

In the case of supernatants of chopped lung preparations, 4 ml were pipetted to a 50 ml test tube.

3.5 g of dipotassium monohydrogen phosphate (anhydrous) and 20 ml of isoamyl alcohol were added to the perchloric acid extract of the chopped lung tissue or to the supernatants of chopped lung preparations. Test tubes were shaken and centrifuged. The organic phase was transferred into a 50 ml test tube containing 4 ml 0.01N hydrochloric acid and 15 ml heptane, shaken and then centrifuged. The organic phase was aspirated and discarded.

3.5 ml of the acid extract were transferred into a 50 ml centrifuge tube containing 0.5 ml 10N sodium hydroxide, mixed well and 2.5 g of sodium chloride and 20 ml chloroform were added. The test tubes were shaken and centrifuged.

The washed extract (top aqueous layer) was transferred to a centrifuge tube, 1.5 g of sodium chloride were added and mixed well, then 20 ml of the washed isoamyl alcohol were added. The test tubes were shaken and centrifuged.

20 ml of the organic phase were transferred to a 50 ml test tube containing 2 ml of 0.1N hydrochloric acid and 15 ml heptane and were shaken and centrifuged. The organic phase was discarded and the aqueous layer was transferred to a small test tube (5 ml capacity).

Fluorophor formation

Fluorophor formation was carried out in 5 ml test tubes. Mixing was carried out after adding each of the following:

0.7 ml sample

0.3 ml 0.1N HCl

0.2 ml 1N NaOH

0.05 ml O-phthaldialdehyde reagent

Exactly four minutes after addition of the O-phthaldialdehyde reagent 0.1 ml of 2M citric acid was added. As fluorophor formation does not occur at acidic pH, fluorophor formation was prevented in blanks by adding citric acid before the O-phthaldialdehyde reagent.

Blank Was prepared by adding the following:

0.7 ml sample

0.3 ml 0.1N HCl

0.2 ml 1N NaOH

0.1 ml 2N citric acid

0.05 ml O-phthaldialdehyde reagent

Fluorescence assay was made using a Farrand MK-1 spectrofluorometer (Farrand Optical Co. Inc., New York) at 358 and 446 mμ excitation and emission wavelengths respectively.

Standard histamine solutions were prepared by dissolving histamine dihydrochloride in 0.1N hydrochloric acid.

Recovery

A standard solution of histamine (1 μg/ml) was prepared with each group of samples. The standard solution was assayed in the same way as the samples. The percentage recovery of histamine was determined.

Specificity

3-Methylindole and 3-methyloxindole are strongly fluorescent

substances. The possibility of interference with the fluorescence assay caused by the presence of these substances was investigated.

2 ml of supernatants from each of experiment 2 (contained 3-methylindole) and 4 (contained 3-methyloxindole) were combined and carried through the extraction procedure and fluorophor formation. Excitation and emission spectra of chopped lung supernatants were examined and compared with an authentic histamine solution (about 50 ng/ml).

Results

Recovery, linearity and specificity

The recovery of histamine from standard solutions is shown in Table 4.4.

The percentage recovery of each experiment was used to correct the histamine estimations on lung tissue and supernatants obtained during that experiment. The mean recovery in six experiments was 80 ± 1.3%.

The fluorescent assay was linear over a wide range of concentrations (from 2 ng/ml to 10 µg/ml).

As shown in Fig. 4.2 samples of supernatants of chopped lung (from preparations containing 3-methylindole and 3-methyloxindole) showed strong peaks having excitation and fluorescence maxima at wavelengths of 358 and 446 mµ respectively. The excitation and emission spectra were identical with those of an authentic solution of histamine.

4.2.9 - Histamine concentration in chopped lung tissue and in supernatants

Incubation of chopped lung with Tyrode solution alone or Tyrode solution containing 3-methylindole or 3-methyloxindole resulted

TABLE 4.4 RECOVERY OF HISTAMINE FROM STANDARD SOLUTIONS CONTAINING 1 µg HISTAMINE IN 1 ml DISTILLED WATER

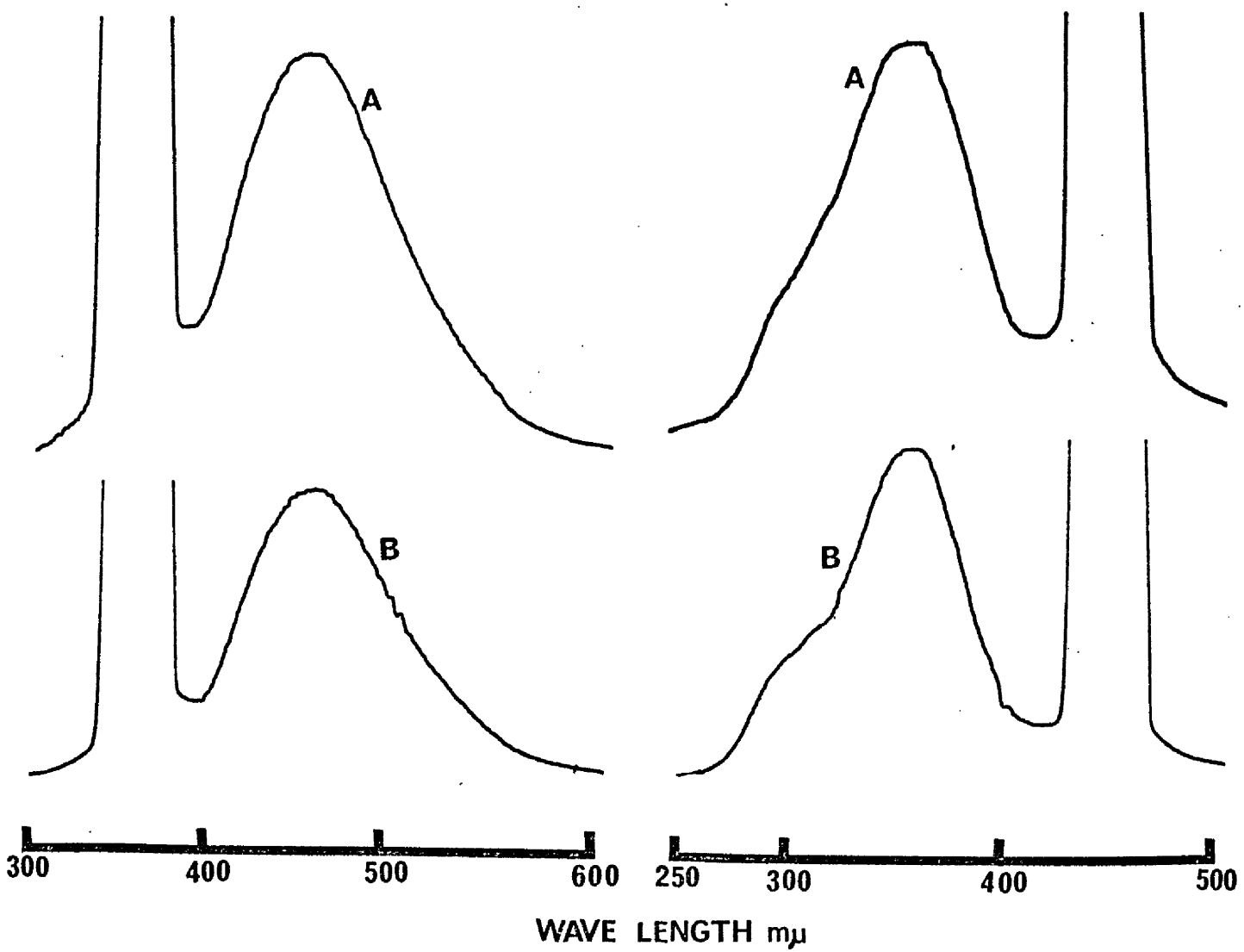
Experiment No.	Recovered histamine	% Recovery
1	0.80	80
2	0.78	78
3	0.82	82
4	0.79	79
5	0.81	81
6	0.80	80
Mean ± S.E.	0.8 ± 0.01	80 ± 1.3

TABLE 4.5 HISTAMINE RELEASE FROM CHOPPED LUNG INCUBATED WITH TYRODE SOLUTION ALONE OR WITH TYRODE SOLUTION CONTAINING 3-METHYLINDOLE (3MI), 3-METHYLOXINDOLE (3MOI) OR COMPOUND 48/80

Experiment No.	Histamine released (µg/g)						Histamine released (%)		
	Tyrode		3MI or 3MOI*		Compound 48/80				
	Total	Supernatant	Total	Supernatant	Total	Supernatant	Tyrode	3MI or 3MOI	Compound 48/80
1	15.32	0.87	15.40	0.84	15.50	3.41	5.70	5.50	22.00
2	10.41	0.21	10.52	0.18	10.30	1.03	2.00	1.80	10.00
3	8.82	0.35	8.70	0.35	8.32	1.49	4.00	4.10	18.00
4	20.51	0.41	20.32	0.38	20.41	4.08	2.00	1.90	20.00
5	12.27	0.98	12.38	0.99	12.23	3.67	8.00	8.00	30.00
6	15.60	0.79	15.75	0.78	15.53	2.33	5.00	5.00	15.00

* 3MI was used in Experiments 1 to 3 and 3 MOI was used in Experiments 4 to 6.

Fig. 4.2 Excitation (right) and fluorescence (left) spectra of histamine extracted from supernatants of chopped lung (A) and of an authentic sample of histamine (B)


A
B
A
B
300
400
500
600
250
300
400
500
WAVE LENGTH mμ

in release of 1.9-8% of total tissue histamine to the supernatant. Incubation in Tyrode solution containing compound 48/80 resulted in release of 10-30% of total tissue histamine to the supernatant. Tissue histamine concentration in calf lungs was variable, ranging from 2.5 to 8.8 µg histamine/g lung tissue.

In all lung samples compound 48/80 consistently released significant amounts of histamine. Incubation with 3-methylindole or with 3-methyloxindole did not increase the release of histamine from chopped calf lung. Table 4.5 shows the release of histamine from chopped calf lung incubated with Tyrode alone, Tyrode containing compound 48/80 and Tyrode containing 3-methylindole or 3-methyloxindole in six experiments.

4.3 - DISCUSSION

4.3.1 - Methodological considerations

The chopped bovine lung preparation has been used most successfully as a test preparation to demonstrate the release of mediators of anaphylaxis by antigen-antibody reactions and by the chemical mediator releaser compound 48/80 (Eyre, 1971a, b, and 1972; Burka and Eyre, 1974b). Other methods for studying the effect of mediator releasers, e.g. measuring changes in tissue and plasma concentrations of histamine during anaphylaxis or using perfused lung preparations or suspensions of leucocytes have often met with little or no success (Aitken, 1970). The wide variation in tissue histamine levels between different calves (Aitken, 1970; Eyre et al, 1973) and in the free histamine concentrations in plasma on repeated sampling from individual animals (Aitken, 1970) made it difficult to draw

conclusions as to changes in plasma and tissue histamine occurring as a result of anaphylaxis (Aitken, 1970; Eyre et al, 1973). In this present study lung tissue from only one animal was used in each experiment to avoid animal to animal variations in tissue histamine concentration.

The bioassay used in this study offers several advantages. It is the only possible method for measuring "slow reacting substance of anaphylaxis", which is considered an important mediator in cattle (Burka and Eyre, 1974a) and a valuable tool when screening for autopharmacodynamically active substances. The sample capacity for prostaglandins, which is relatively an important mediator in bovine anaphylaxis (Burka and Eyre, 1974a), was increased by extracting prostaglandins in ethyl acetate and resuspending them in a smaller volume of perfusion fluid. Another advantage of this technique is that it gives almost complete recovery for prostaglandins and extracts only traces of other pharmacologically active substances (Gilmore et al, 1968).

It has been shown that sodium meclofenamate, which is a broad spectrum mediator antagonist (Burka and Eyre, 1974a), is capable of protecting calves against the 3-methylindole induced anaphylactoid reaction (Atkinson et al, 1977). This strongly suggests the possible involvement of several mediators in the pathogenesis of the 3-methylindole induced pulmonary disease. The guinea pig ileum and oestrus rat uterus offer a model sensitive to a wide range of mediators of anaphylaxis. The guinea pig ileum is the recommended preparation for the bioassay of "slow reacting substances of anaphylaxis"

(Chakravarty, 1959), and histamine (Vugman and Rocha E. Silva, 1966). The isolated rat uterus and isolated guinea pig ileum, on which 5-hydroxytryptamine causes contraction, have been used most frequently as a test object for 5-hydroxytryptamine (Offermeier and Ariens, 1966). A survey by Orange and Austen (1969) showed that the oestrus rat uterus can be used for the bioassay of bradykinin and prostaglandins E_1 and $F_{2\alpha}$.

3-Methylindole in the concentration used in the present work (2 µg/ml) was found not to interfere with the response of the guinea pig ileum to histamine, and it would be reasonable to assume that 3-methylindole, in small concentrations, does not interfere with the bioassay of other mediators.

4.3.2 - Failure of 3-methylindole and 3-methyloxindole to contract bovine pulmonary smooth muscles in vitro

It has been reported that 3-methylindole causes contraction of the bovine and relaxation of the ovine pulmonary vein in vitro (Eyre, 1975). Based on these findings and on species differences in response to orally administered L-tryptophan, which causes pulmonary lesions in cattle, but not in sheep (Carlson et al, 1968), Eyre (1975) suggested that the mechanism of action of 3-methylindole involves a direct action on the bovine pulmonary vein (Eyre, 1975). In this present study 3-methylindole and 3-methyloxindole (5-640 µg/ml) had no effect on isolated bovine pulmonary smooth muscle preparations. Apart from failure of this present study to confirm Eyre's (1975) observations, the findings of Bradley et al (1978) that 3-methylindole induces pulmonary damage in sheep similar to that in cattle, whereas

Eyre (1975) has shown that 3-methylindole relaxes the ovine pulmonary vein, also argues against Eyre's (1975) hypothesis. Therefore the apparent difference in susceptibility to L-tryptophan induced disease in these species does not reflect a difference in target organ response, but is probably due to difference in the quantity of 3-methylindole produced from L-tryptophan in the rumen (Bradley et al, 1978).

It should be noted that the report of Eyre (1975) does not state the vehicle used for addition of 3-methylindole to isolated organ preparations and since 3-methylindole is not sufficiently soluble in water and electrolyte solutions to give the concentrations used, an action caused by the unspecified solvent for 3-methylindole cannot be excluded.

Concentrations of 3-methylindole used by Eyre (1975) were considerably higher than those found in plasma of 3-methylindole dosed cattle displaying symptoms of severe acute respiratory distress. Concentrations of 3-methylindole and 3-methyloxindole never exceeded 35 µg/ml and 17 µg/ml respectively in plasma of 3-methylindole dosed cattle (Chapter 3). Concentrations of 3-methylindole and 3-methyloxindole used in this present study ranged from 5 to 640 µg/ml.

4.3.3 - Failure of 3-methylindole and 3-methyloxindole to release mediators of anaphylaxis from the chopped bovine lung

The supernatants of chopped bovine lung caused contraction of the guinea pig ileum. The contractions of the guinea pig ileum in the case of supernatants of chopped lung incubated with Tyrode

solution alone or Tyrode containing 3-methylindole or 3-methyloxinoole were due solely to the presence of histamine in the incubation media since they were blocked completely by the antihistamine mepyramine. Incubation of chopped lung with 3-methylindole or 3-methyloxindole did not increase the guinea pig ileum contracting activity of the supernatants of incubation media. Results of the spectrofluorimetric determination of histamine indicate that incubation of chopped lung with 3-methylindole or 3-methyloxindole does not cause the release of histamine. Chopped lung preparations incubated with 3-methylindole or 3-methyloxindole released quantities of histamine similar to those released spontaneously from chopped lung preparations incubated with Tyrode alone. The presence of 3-methylindole or 3-methyloxindole did not cause additional release of histamine. The experimental system was sufficiently sensitive to demonstrate clearly the release of mediator substances from chopped lung by compound 48/80. Incubation of chopped lung with compound 48/80 consistently increased the concentration of histamine and guinea pig ileum contracting activity of supernatants of chopped lung preparations. Contractions caused by these supernatants were only partially antagonized by mepyramine. Thus compound 48/80 caused not only the release of histamine but also released other mediator substances. The nature of these mediators was not identified. It has been shown that compound 48/80 releases histamine (Eyre, 1971a) and 5-hydroxytryptamine (Eyre, 1972), both of which cause contraction of the guinea pig ileum, and dopamine (Eyre, 1971b) from the chopped bovine lung. No evidence was obtained for the release of "slow reacting substance of anaphylaxis"

a putative mediator of bovine anaphylaxis (Burka and Eyre, 1974a, b) using the chopped lung preparations. The isolated guinea pig ileum is the recommended test preparation for bioassay of "slow reacting substance of anaphylaxis" (Chakravarty, 1959).

The release of "slow reacting substance of anaphylaxis", histamine, 5-hydroxytryptamine and dopamine, from sensitized chopped bovine lung, after exposure to specific antigen, has been demonstrated (Eyre, 1971a, b, 1972; Burka and Eyre, 1974b).

Lung tissue concentration of histamine and spontaneous release of histamine detected in this study (Table 4.5) varied between different animals consistent with the results of previous work in cattle (Aitken, 1970; Eyre, 1971a).

4.3.4 - Mediators of anaphylaxis and pulmonary veno-constriction as aetiological factors in bovine pulmonary disease

It has been hypothesized that the pulmonary vein is a primary target tissue in bovine anaphylaxis (Eyre, 1971c) and in naturally occurring and experimentally induced acute pulmonary bovine oedema and emphysema (Breeze, 1973; Eyre, 1975). This hypothesis holds that contraction of the pulmonary vein, in response to tryptamine analogues absorbed from dietary tryptophan or due to the action of mediator substances released into the blood as a result of antigen-antibody interaction, would increase capillary hydrostatic pressure enhancing transudation from the pulmonary capillary bed leading to pulmonary oedema. This hypothesis is based on results of <u>in vitro</u> studies indicating that the pulmonary vein is considerably more

sensitive than the artery to the effect of mediators of anaphylaxis (Eyre, 1975; Burka and Eyre, 1977b). Also since pulmonary oedema is a feature both of anaphylaxis (Aitken and Sanford, 1969b) and naturally and experimentally induced acute bovine pulmonary emphysema, this hypothesis has been favoured (Breeze, 1973). Pulmonary venous constriction which causes increased capillary hydrostatic pressure would more readily explain the development of pulmonary congestion and oedema than pulmonary arterial spasm which would tend to have the opposite effect on the capillary bed. However evidence from other studies on pulmonary haemodynamics argue against this hypothesis.

Firstly, experimental haemodynamic manoeuvres involving obstruction to the left side of the heart have been unsuccessful in the production of pulmonary oedema. Briefly Dickinson (1976) stated: "It is not possible to produce pulmonary oedema in the rabbit, or in any other mammals by any obstruction to the left side of the heart, even with a ligature tied round the proximal aorta. All that happens is that the animal dies of acute circulatory failure - what used to be described as 'forward failure' of the heart. The lungs are not pathologically engorged with blood at necropsy. Partial degrees of left ventricular or aortic obstruction are no more successful. Pulmonary oedema can nonetheless easily be produced in the rabbit, as in other mammals, by sufficiently large and rapid infusions of blood, plasma or saline - most easily with saline". Because the normal adult pulmonary vessels are highly distensible any increase in driving pressure or in terminal pressure e.g. as a result of pulmonary veno-constriction would result in a precipitous fall of

pulmonary vascular resistance (Williams, 1954; Borst, Berglund and McGregor, 1957). Therefore pulmonary venous obstruction tends to result in only a small rise in arterial pressure, a probable lowering of resistance and a high risk of cardiac failure.

Secondly, although pulmonary oedema was observed following administration of mediator substances in cattle (Hull, 1965; Aitken and Sanford, 1972) this oedema was only transient. Rapid recovery was observed following administration of histamine, bradykinin, 5-hydroxytryptamine and prostaglandin E_1, E_2 and $F_{2\alpha}$ (Aitken and Sanford, 1972; Burka and Eyre, 1974a). The effect of mediators of anaphylaxis on the bovine lung requires further investigation. Detailed studies in the dog have shown that histamine, bradykinin, endotoxin and compound 48/80 cause bronchial venules to become highly permeable to larger molecules without apparent effect on pulmonary capillary permeability. The period of increased venular permeability is transient. Leakage consistently stopped within 10 minutes after histamine or bradykinin administration (Peitra, Szidon, Leventhal and Fishman, 1971; Pietra, Szidon, Carpenter and Fishman, 1974). The mechanism by which histamine and bradykinin exert this selective effect on the endothelium of the bronchial venules is speculative, the most attractive being the idea of opening interendothelial junctions by contractions of the endothelial fibrils with which bronchial venular endothelium is richly endowed (Pietra et al, 1974).

Thirdly, the effects reported for mediators of anaphylaxis on pulmonary haemodynamics are inconsistent. In the bovine Burka and Eyre (1974a) and Hull (1965) reported a fall in pulmonary

arterial pressure following administration of histamine, while Aitken and Sanford (1972), Lewis and Eyre (1972b) and Desliens (1958) reported a rise. Similar results were obtained with other species. In human subjects a dose of histamine which was large enough to cause a fall in systemic blood pressure had no effect on the pulmonary circulation (Nemir, Stone, Mackrell and Hawthorne, 1954). Similar observations were reported by Spitzbarth, Gersmeyer, Weyland and Gasteyer (1957) whereas others obtained either a decrease (Helander, Lindell, Soderholm and Westling, 1962; Westling, 1963), or a rise (Lambertini, Lanari and Zubiaur, 1960) in pulmonary arterial pressure. The effects of histamine in dogs (Friedlberg, Katz and Steinitz, 1943; Peters and Horton, 1944; Delaunois, Kordecki, Polet and Ryzewski, 1959; Paulet and Bernard, 1963) also revealed trends similar to those observed in man. The effects of 5-hydroxytryptamine on the pulmonary arterial pressure of the bovine is equally inconsistent. Burka and Eyre (1974a) reported reduction in pulmonary arterial pressure following injection of 5-hydroxytryptamine in calves, while Aitken and Sanford (1972) and Lewis and Eyre (1972b) recorded an increase. Continuous infusion of 5-hydroxytryptamine caused increased pulmonary arterial pressure in dogs (Rudolph and Paul, 1957; Aviado, 1960) and its direct injection into the pulmonary artery caused marked vaso-constriction (Rose and Lazaro, 1958). The principal site of the increased resistance to flow through the lungs appeared to be in the precapillary vessels. Oedema was not observed even after repeated doses of 5-hydroxytryptamine (Shepherd, Donald, Linder and Swan, 1959).

Despite the expected vaso-constrictor effect of 5-hydroxy-tryptamine, chronic injection of the drug in rats was not regarded as a useful method for the production of pulmonary hypertension. Pressure recordings gave inconsistent right ventricular systolic pressure between 9 and 43 mm Hg (Blank, Miller and Dammann, 1961).

The effect of bradykinin on pulmonary arterial pressure is also inconsistent. Burka and Eyre (1974a) reported reduction while Aitken and Sanford (1972) reported a rise in pulmonary artery pressure following infusion of bradykinin in calves. In dogs bradykinin caused a fall in both systemic and pulmonary arterial pressure (Maxwell, Elliott and Kneebone, 1962; Rowe, Afonso, Castillo, Lloy, Lugo and Crumpton, 1963). In human subjects it was concluded that bradykinin causes no significant changes in pulmonary vascular resistance.

The effects of prostaglandins on pulmonary arterial pressure are also variable. Prostaglandin E_1, E_2 and $F_{2\alpha}$ were reported to increase pulmonary arterial pressure (Lewis and Eyre, 1972[a]; Burka and Eyre, 1974a; Aitken and Sanford, 1975), while Aitken and Sanford (1975) and Burka and Eyre (1974a) obtained a reduction following prostaglandin E_1 and E_2 infusion in calves. In dogs prostaglandin E_1 caused mild active vasodilation while prostaglandin $F_{2\alpha}$ resulted in mild active vaso-constriction in the lung. Prostaglandin E_1 and A_1 caused passive shrinkage of the pulmonary vascular bed secondary to a shift of blood to the peripheral circulation (Nakano and McCurdy, 1968; Alpert, Haynes, Knuston, Dalen and Dexter, 1973). Nakano and Cole (1969) have consistently observed increases in pulmonary arterial pressure together with decreases in pulmonary resistance in dogs with

intravenous injection of prostaglandins E_1 and A_1. Other workers (Hauge, Lunde and Waaler, 1966; Maxwell, 1967) have found no significant changes in pulmonary arterial pressure during intravenous infusion of prostaglandin E_1.

Most of the conclusions concerning the actions of mediators of anaphylaxis on the pulmonary circulation have been made on the basis of pulmonary artery pressures and cardiac outputs alone. The results of these studies have varied from investigator to investigator. Yu (1969) has pointed out the necessity of measuring simultaneous pressure and volume in determining whether an agent or manoeuvre has an active or merely a passive effect on the pulmonary vasculature. Thus parallel increases or decreases in pulmonary distending pressure and pulmonary blood volume represent passive vascular distension or shrinkage usually secondary to major changes in cardiac or peripheral vascular haemodynamics. Changes in opposite directions by pressure and capacity parameters are indicative of active pulmonary vasomotion (Yu, 1969).

The evidence supporting the local pulmonary vaso-constrictor effects of mediators of anaphylaxis are derived from perfusion experiments of lung, and from studies on isolated pulmonary smooth muscle preparations. In the bovine, increase in pulmonary perfusion pressure and ventilation resistance was reported following infusion of the isolated lung with histamine, 5-hydroxytryptamine (Lewis and Eyre, 1972b; Eyre et al, 1973) and bradykinin (Eyre et al, 1973). Prostaglandin $F_{2\alpha}$ produced only slightly increased arterial perfusion pressure; air overflow was not affected even at high doses.

Prostaglandin E_1 and E_2 reduced the resistance to perfusion, but airflow was unaltered (Lewis and Eyre, 1972a). Purified "slow reacting substance of anaphylaxis" caused contraction of the pulmonary vein, tracheal muscle, bronchial preparation, but not the pulmonary artery (Burka and Eyre, 1977b). Prostaglandin $F_{2\alpha}$ caused contraction of the pulmonary artery, vein and bronchus, prostaglandin E_1 relaxed the artery and bronchus, while prostaglandin E_2 caused contraction of the pulmonary artery and bronchus or at higher concentrations relaxation of the contracted bronchus (Burka and Eyre, 1974c; Aitken and Sanford, 1975). The effect of prostaglandin E_1 and E_2 on the pulmonary vein is inconclusive. Aitken and Sanford (1975) reported that prostaglandin E_1 and E_2 caused contraction of the pulmonary vein, while Burka and Eyre (1974c) reported that prostaglandin E_1 consistently evoked dose-related relaxation of the vein and prostaglandin E_2 caused relaxation at low concentration and induced contraction at higher concentrations.

Histamine and 5-hydroxytryptamine cause contraction of the bovine pulmonary vein, artery, tracheal muscle and bronchus (Aitken and Sanford, 1970; Eyre, 1971c and 1975).

Further evidence against the possible implication of pulmonary veno-constriction as a primary event in the pathogenesis of the 3-methylindole induced pulmonary disease is that the spasmogenic activity of different tryptamine analogues on the bovine pulmonary vein does not correlate with the ability of individual tryptamine analogues to produce pulmonary disease. Only 3-methylindole produced acute pulmonary oedema and emphysema in cattle, while stronger vaso-

active amines, tryptamine and 5-hydroxytryptamine, did not (Jarvie et al, 1977). Furthermore post-mortem examination of calves, after fatal doses of the chemical mediator releaser compound 48/80 revealed congestion and engorgement of the liver in some calves, but no other gross anatomical changes were observed (Lewis and Eyre, 1972b).

In conclusion an effect on the pulmonary vein or the "non-cytotoxic" release of mediators of anaphylaxis is not enough to explain the pulmonary damage caused by 3-methylindole. Results of recent work have shown ultrastructural evidence of early cell injury to the membranous pneumocyte preceding the development of pulmonary oedema after administration of 3-methylindole in goats (Huang et al, 1977). Thus pulmonary oedema may not be a primary lesion in the 3-methylindole induced pulmonary disease. The disease process starts with specific cellular injury probably as a result of cytochrome P-450 mediated metabolic activation of 3-methylindole to a highly electrophilic metabolite (Chapter 6). This specific cellular injury stimulates the release of mediators of anaphylaxis("cytotoxic release") causing immediate anaphylactoid reaction, and provokes an inflammatory reaction in the lung. This hypothesis is consistent with: a) Meclofenamate protects calves against the acute effects of 3-methylindole (Atkinson et al, 1977); b) The sequence of ultrastructural changes showing that signs of cellular injury occurs as early as two hours after 3-methylindole administration. Meticulous examination of epithelial and endothelial cell junctions has failed to identify unique changes in alveolar capillary membranes, except damaged membranous pneumocytes, that could be indicted as the initial event in a pathogenetic

sequence leading to lung oedema in 3-methylindole dosed goats and it was concluded that the pulmonary oedema may represent the vascular phase of the inflammatory response to membranous pneumocyte injury (Huang et al, 1977). In the dog detailed ultrastructural and physiological studies have failed to elucidate the mechanism of pathogenesis of pulmonary oedema after endotoxin shock and it was concluded that lung injury after endotoxin shock cannot be attributed to increased capillary permeability as a primary event (Peitra et al, 1974).

c) Results of the present work indicate that 3-methylindole and 3-methyloxindole have no direct effect on bovine pulmonary smooth muscles excluding the possibility of a primary hydrodynamic imbalance in the normal equilibrium of forces across the alveo capillary membrane resulting in excess filtration over reabsorption.

d) Metabolism of 3-methylindole by cytochrome P-450 dependent mixed function oxidase involves the formation of reactive metabolites which arylate cellular macromolecules (Chapter 6). Alkylating and arylating foreign compounds are endowed with potent specific cytotoxic effects and are capable of causing cell necrosis.

Chemical substances which consistently produce lung damage when administered by routes other than inhalation are relatively rare. The mechanism of action of these pneumotoxic substances is poorly understood. A current view is that pulmonary oedema and emphysema produced by these substances represent secondary events.

CHAPTER 5

EFFECTS OF 3-METHYLINDOLE AND 3-METHYLINDOLE ANALOGUES ON ANAESTHETIZED CALVES

5.1 INTRODUCTION

The studies presented in Chapter 6 suggest that the pulmonary toxic effect of 3-methylindole is probably due to its metabolic activation to a highly electrophilic metabolite. However the mechanism of metabolic activation of 3-methylindole remains unclear. Direct approach by chemical isolation and identification of reactive metabolites is probably not feasible because of the instability of the metabolite. However an investigation into the structure-activity relationship of 3-methylindole analogues and metabolites would assist in understanding the mechanism of metabolic activation of 3-methylindole.

In this present work the acute pulmonary effects of 3-phenylindole, 5-methylindole, 7-methylindole and 3-methyloxindole are investigated and compared with those produced by 3-methylindole.

Other workers have shown that 3-methylindole produces acute pulmonary toxic effects which can be measured by physiological recording systems. Immediately after the infusion of small doses of 3-methylindole calves displayed signs of respiratory embarrassment, pulmonary arterial hypertension and systemic hypotension (Atkinson et al, 1977). Previous work on other 3-methylindole analogues, viz indoleacetic acid (intravenously), indole, tryptamine and 5-hydroxytryptamine (orally) indicate that these compounds do not produce acute pulmonary emphysema in cattle (Pirie et al, 1976; Jarvie et al, 1977; Hammond, Carlson and Breeze, 1980).

5.2 EXPERIMENTAL

5.2.1 - Materials

3-Methyloxindole was prepared as described in Chapter 3.

Other chemicals were obtained from commercial sources as described previously.

5.2. - Animals

Six calves (Ayrshire breed), 60 to 100 kg body weight, were designated 1 to 6. Food was withheld 12 hours before experiments.

5.2.3 - Recording and measurement of physiological parameters

Calves were anaesthetized by intravenous injection of sodium pentobarbitone (Abbott Ltd.) as 6% solution at a dose rate of 20 mg/kg, with additional 2 mg/kg doses intravenously given as required throughout the course of the experiments to maintain anaesthesia. A cannula was introduced into the rumen to prevent the risk of ruminal tympany.

An endotracheal tube was introduced through the mouth and was attached via a Fleisch pneumotachograph (Metabo Epalinges, Lausanne) to a differential pressure transducer. From the record obtained respiratory rate could be read and changes in respiratory depth were measured. Heart rate was measured via a Neilson instantaneous heart rate meter (Devices Ltd.) driven by a signal from an electrocardiograph (Devices Ltd.) using standard limb lead II. Polythene or nylon catheters and cannulae (Portex Ltd., external diameter of 2 to 3mm) were used to catheterize blood vessels to monitor blood pressure changes. The cannulae were filled with anticoagulant heparin solution (Boots Pure Drug Co.) containing 1000 units per ml before insertion into the blood vessels. Systemic blood pressure was measured from a cannula introduced into a carotid artery and connected to a Bell and Howell pressure transducer (Type 4-422-0001).

The pressure transducer was adjusted to the elevation of the anaesthetized animal and calibrated before each experiment by means of a mercury manometer.

Pulmonary blood pressure was measured similarly after catheterization of the pulmonary artery. This was carried out by introducing a catheter into the jugular vein until a length of catheter approximately equal to the distance between the point of insertion in the neck and the third rib had been passed into the vein. At this point the catheter was judged to have reached the heart. Examination of the pressure changes being recorded allowed assessment of passage of the catheter on into the right ventricle and finally into the pulmonary artery. In every case the position of the catheter was verified at post-mortem examination. All parameters were measured using an 8-channel physiological pen recorder (Type M19 Devices Ltd.)

Mean arterial pressure was calculated from the formula $MAP = Pd + \frac{Ps - Pd}{3}$ where Pd = mean diastolic pressure and Ps = mean systolic pressure.

Infusions were made into a recurrent tarsal vein via an indwelling cannula. 5 ml of saline containing 10 units heparin per ml were injected following administration of each dose. All infusions were administered over a period of one minute with a subsequent dose given after one hour.

5.2.4 - Preparation of solutions of 3-methylindole and 3-methylindole analogues

All indolic compounds were administered as a 20 mg/ml solution in 10% "Cremophor-EL" in physiological saline. This solvent dissolves

3-methylindole and 3-methylindole analogues well, is miscible with water and plasma and 3-methylindole analogues do not precipitate out on infusion.

Propylene glycol, the solvent used for administration of 3-methylindole by other workers (Carlson et al, 1975), has been shown to produce significant cardiorespiratory changes and haemolysis in calves (Gross, Kitzman and Adams, 1979) and is unsuitable for rapid intravenous administration because of its high viscosity.

Results

Effects of "Cremophor-EL"

A dose of the solvent (10% "Cremophor-EL" in physiological saline solution) equal to that used to deliver the dose of 3-methylindole analogues (30-50 ml) caused a small (about 5 mm Hg) transient (lasting for 1-3 minutes) rise in pulmonary arterial blood pressure in Calves 1, 2 and 3.

Effect of 3-methylindole and 3-methylindole analogues

Doses of 10 mg/kg of 3-methyloxindole, 3-phenylindole, 5-methylindole and 7-methylindole caused rises in pulmonary arterial blood pressure. This was transient, lasting for 1-3 minutes after which pulmonary arterial pressure returned to pre-injection values. No other changes were observed during a one hour observation period after the administration of each compound. All calves reacted to 3-methylindole. Administration of a dose of 1 mg/kg of 3-methylindole was followed (within 1-2 minutes) by simultaneous onset of pulmonary arterial hypertension, a marked fall in carotid arterial pressure, apnoea followed by prolonged period of tachypnoea and hyperpnoea.

TABLE 5.1 EFFECTS OF 3-METHYLINDOLE (3MI), 3-METHYLOXINDOLE (3MOI), 3-PHENYLINDOLE (3PI), 5-METHYLINDOLE (5MI) AND 7-METHYLINDOLE (7MI) ON ANAESTHETIZED CALVES

Calf No.	A Compound	Dose (mg/kg)	Effects on B Respiration	C Pulmonary artery pressure	D Carotid pressure
1	3MOI	10	0	5	0
	3MI	1	50	13	80
2	3MOI	10	0	5	0
	3MI	1[E]	100	15	120
3	3MOI	10	0	6	0
	3MI	1[E]	100	13	106
4	5MI	10	0	0	0
	3PI	10	0	5	0
	7MI	10	0	0	0
	3MI	1	71	15	60
5	7MI	10	0	0	0
	5MI	10	0	10	0
	3PI	10	0	0	0
	3MI	1	40	10	40
6	3PI	10	0	7	0
	5MI	10	0	0	0
	7MI	10	0	0	0
	3MI	1	60	15	80

A - Compounds administered as the order listed, with next compound injected after one hour. Intravenous infusions were made over one minute.

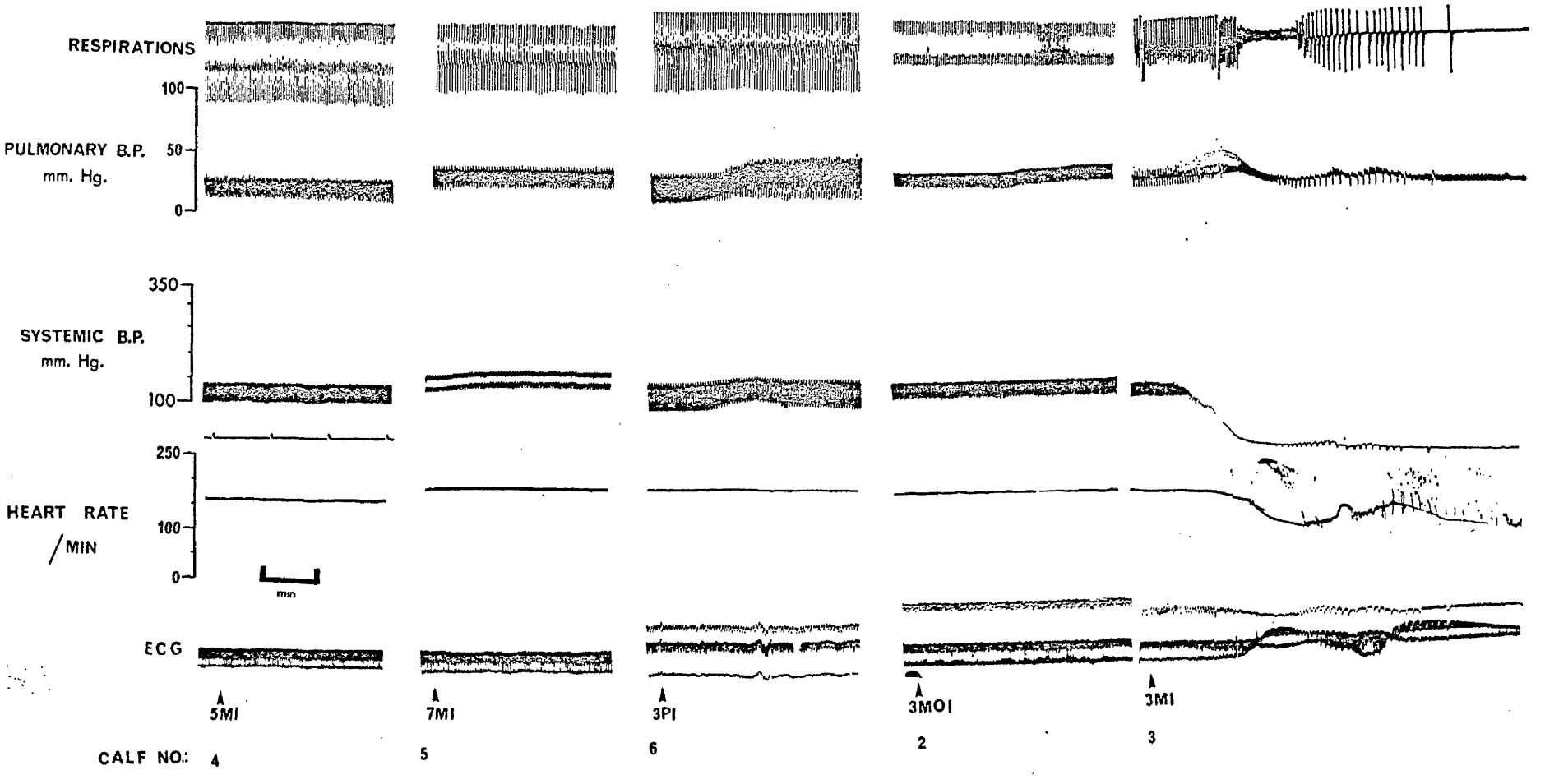
B - Respiration: Respiratory depth maximal decrease (%).

C - Pulmonary artery pressure: Mean pulmonary arterial pressure, maximal increase (mm Hg).

D - Carotid pressure: Mean carotid arterial blood pressure, maximal decrease (mm Hg).

E - Injections made over 0.5 minute period, followed by death within two minutes.

Figure 5.1 Effects of 3-methylindole (3MI), 3-methyloxindole (3MOI), 3-phenylindole (3PI), 5-methylindole (5MI) and 7-methylindole (7MI) on anaesthetized calves


RESPIRATIONS
100
PULMONARY B.P.
mm. Hg.
50
0
350
SYSTEMIC B.P.
mm. Hg.
100
250
HEART RATE
/MIN
100
0
min
ECG
5MI
7MI
3PI
3MOI
3MI
CALF NO.: 4
5
6
2
3

Animals which died showed a recurrence of apnoea about 1-2 minutes after the initial period of apnoea, making irregular gasping respiratory efforts before death. Table 5.1 and Fig. 5.1 show the effects of 3-methylindole analogues on six anaesthetized calves.

5.3 DISCUSSION

Apart from a small rise in pulmonary arterial blood pressure which can, at least in part, be attributed to the solvent, no toxic effects were noticed after the rapid administration of doses of 10 mg/kg body weight of 3-methyloxindole, 5-methylindole, 7-methylindole or 3-phenylindole intravenously in calves. 3-Methylindole in a much smaller dose (1 mg/kg body weight) was extremely toxic causing pulmonary hypertension, systemic hypotension, respiratory embarrassment and even death. Intravenous doses of 3-methylindole were delivered over a period of one minute in four calves, and the calves survived 3-methylindole infusions. When the same dose of 3-methylindole was given over a shorter period (0.5 minutes) in two calves, it was followed by death. Previous work on 3-methylindole (Atkinson et al, 1977) reported similar effects to those reported here.

5-Methylindole, 7-methylindole, 3-methyloxindole and 7-phenylindole did not produce the acute pulmonary toxic effects of 3-methylindole. It has been shown that the metabolism of indole involves two routes, a major route through conversion to indoxyl and a minor route through oxindole formation(King et al, 1966). The formation of indoxyl from 3-methylindole is blocked by the 3-methyl group. Thus the effect of the 3-methyl group is to shift the metabolism of 3-methylindole towards the oxindole route (Hammond,

Carlson and Willett, 1979). 7-Methylindole and 5-methylindole would be expected, like indole, to be metabolized mainly through indoxyl formation. Failure of 3-methyloxindole, the major metabolite of 3-methylindole (Chapter 3), to produce acute pulmonary toxicity is consistent with the finding that 3-methyloxindole does not become enzymatically converted to reactive metabolite(s) (Chapter 6). Thus the pulmonary toxicity of 3-methylindole in cattle involves a metabolite intermediate between 3-methylindole and 3-methyloxindole or, less likely, a metabolite produced by some minor route not involving 3-methyloxindole. Work with furano compounds indicates that the furan moiety of these compounds is essential for the pulmonary toxic effect produced by certain members of this group of compounds, viz 3-methylfuran and 4-ipomeanol. Replacing the furan moiety with a methyl or a phenyl substituent resulted in loss of both the pulmonary toxic effect and the ability of these compounds to become enzymatically converted to reactive metabolites (Boyd, Burka, Wilson and Sasame, 1978; Boyd and Burka, 1978). Different furano compounds exhibit specificity towards different organs, e.g. furosemide produces hepatic and renal lesions (Jollow and Mitchell, 1973; Mitchell, Potter and Jollow, 1973) whereas 3-methylfuran and 4-ipomeanol cause lung lesions (Boyd, 1976; Boyd, Statham, Franklin and Mitchell, 1978). Therefore results of this present work should be interpreted with caution. Indolic compounds failing to produce acute pulmonary toxicity may cause extrapulmonary lesions. Results of recent work (Hammond, Carlson and Breeze, 1980) have shown that indole produces renal lesions in cattle. The nephrotoxic effect of indole was

attributed to haemoglobinuric nephrosis resulting from haemolysis which was observed after oral administration of indole in cattle. The possibility that the nephrotoxic effect of indole is, like, furosemide, caused by a reactive metabolite (Mitchell, Potter and Jollow, 1973) should be evaluated.

CHAPTER 6

THE CONVALENT BINDING OF 3-METHYLINDOLE TO BOVINE TISSUES

6.1 INTRODUCTION

6.1.1 - Metabolic activation of foreign compounds to cytotoxic chemically reactive metabolites

Drugs and other foreign compounds are most frequently metabolized by the host organism in order that they may be excreted more easily. In general metabolism converts parent drugs into less lipid soluble compounds. These less lipid soluble metabolites are usually more water soluble and most frequently less pharmacologically active than the parent drug. In some instances, however, metabolites are more pharmacologically active and in some instances are more toxic than the parent compound. Where metabolism leads to increased activity (whether toxic or pharmacologically valuable) in the majority of cases the metabolite combines reversibly with action sites in tissues. In a number of instances, however, drugs and other foreign compounds become converted to chemically reactive metabolites.

The concept of metabolic activation of drugs and foreign compounds to chemically reactive metabolites was first introduced by Miller and Miller (1966) and Magee and Barnes (1967) to explain the mechanism of carcinogenicity of certain chemicals. This hypothesis holds that chemicals bring about their carcinogenic effect by combining covalently with nucleic acids and other tissue macromolecules or become converted to chemically reactive metabolites which in turn combine covalently with nucleic acids (Miller and Miller, 1966; Magee and Barnes, 1967; Miller, 1970; Weisburger and Weisburger, 1973). More recently the concept of metabolic activation

of foreign compounds to chemically reactive metabolites has been further extended to explain the production of tissue necrosis produced by certain toxic compounds. Chemically reactive metabolites of these compounds produce cellular necrosis by combining with a variety of cellular macromolecules essential for life (Gillette et al, 1974). Evaluation of this hypothesis has been difficult; some new pharmacokinetic principles had to be introduced. Conventional pharmacokinetic studies deal with examples in which the parent drug molecule or pharmacologically active metabolite produce its effects by combining reversibly with receptor sites. In this case the response is usually proportional to the drug or active metabolite plasma concentration. Thus their pharmacological activity can be evaluated by measuring the concentration of the drug or the active metabolite in plasma (Baggot, 1977a). However when the response is tissue damage caused by covalent binding of chemically reactive metabolites to tissue macromolecules, it would not be logical to expect a relationship between the plasma level of the active metabolite and the severity of the lesions (Gillette et al, 1974). Furthermore with highly reactive metabolites, little or none reaches the plasma, since much of the metabolite often decomposes, reacts with cellular constituents, or is further metabolized before it can be isolated in body fluids or urine (Mitchell and Jollow, 1974). One approach to the problem would be to determine whether radiclabelled toxic substances become covalently bound to macromolecules in tissues which become necrotic and to evaluate the correlation between the severity of lesions in target tissues, and the amount of covalently bound metabolites (Gillette, 1974a, b).

6.1.2 - Target macromolecules for covalent binding with reactive metabolites

The specificity of reactive metabolites for binding covalently to a specific target substance depends on the chemical nature of the reactive metabolite being studied (Gillette, 1974a, b). Some reactive metabolites particularly those having relatively low chemical reactivities are extremely specific and become preferentially bound to certain macromolecules by first combining reversibly with active centres on a specific macromolecule to form a complex that rearranges to form a covalently bound conjugate. This mechanism is the basis of the specific inhibition of choline esterases by organophosphate insecticides. With highly reactive metabolites, however, it is difficult to identify target macromolecules because they react with many different cellular constituents including proteins, lipids and nucleic acids and lead to diverse toxicities (Miller and Miller, 1966; Magee and Barnes, 1967; Miller, 1970; Weisburger and Weisburger, 1973). It would be difficult to determine whether cellular toxicity results from the covalent binding of the reactive metabolite to a single kind of macromolecule or from the concerted action originating from multiple biochemical lesions caused by the covalent binding of the reactive metabolite to several kinds of cellular macromolecules. Therefore there is little value in selecting a particular type of macromolecule as the basis of a general test system for determining whether a toxicity caused by a given foreign compound is mediated by a reactive metabolite. It is not sufficient to demonstrate covalent binding to cellular macromolecules of a chemically reactive

metabolite-forming toxic foreign compound. Binding could occur to cellular macromolecules not essential to cell life or function or the biochemical lesions caused by covalent binding of the reactive metabolite may be rapidly recognized and repaired by repair mechanisms (Gillette, 1974a, b). For toxicity to be considered the result of covalent binding of reactive metabolites the toxicity should be roughly proportional to the number of target macromolecule-reactive metabolite conjugates formed in the tissue after administration of the toxic substance. Further, in the presence of drug-metabolizing enzyme inducers and inhibitors, the incidence and severity of lesions should also parallel the extent of covalent binding to cellular macromolecules if the reactive metabolite is to be considered the cause of the toxicity (Gillette, 1974a, b).

6.1.3 - Hepatotoxic drugs as models for the investigation of the mechanism of foreign compound induced tissue injury

The majority of current knowledge on the toxicity of reactive metabolites derives from studies made with hepatotoxic drugs in particular halobenzenes and acetaminophen. Additionally comparative studies on the hepatotoxicity of pyrrolizidine alkaloids represent an example of a naturally occurring toxic hazard to grazing animals. The studies on these three groups of compounds are briefly reviewed here.

1) Halobenzenes

A - Correlation between covalent binding and severity of toxicity

Halobenzenes are relatively chemically inert compounds and do not react covalently with tissue macromolecules. However halobenzenes undergo metabolic activation by cytochrome-P450 dependent mixed function

oxidases to a highly reactive, electrophilic, intermediate which in turn reacts covalently with nucleophilic sites on cellular macromolecules of hepatic cells causing cellular necrosis (Brodie, Reid, Cho, Sipes, Krishna and Gillette, 1971; Jollow, Mitchell, Zampaglione and Gillette, 1972; Jollow, Mitchell, Zampaglione and Gillette, 1974). Thus pre-treatment of rats with phenobarbital, which induces the activity of cytochrome P-450 in the liver, increases the covalent binding of radiolabelled bromobenzene to liver tissue proteins, the severity of the liver necrosis and also increases the rate of disappearance of bromobenzene from the body (Reid, Christie, Krishna, Mitchell, Moskowitz and Brodie, 1971; Reid and Krishna, 1973; Zampaglione, Jollow, Mitchell, Stripp, Hamirck and Gillette, 1973). In contrast pre-treatment of rats with SKF 525-A or piperonyl butoxide compounds which inhibit microsomal drug metabolizing enzymes in the liver, decrease both covalent binding of radiolabelled bromobenzene to liver proteins and severity of the liver necrosis and decrease the rate of disappearance of bromobenzene from the body after administration of radiolabelled bromobenzene from the body (Mitchell et al, 1971; Reid et al, 1971; Jollow et al, 1972; Reid and Krishna, 1973; Zampaglione et al, 1973). Thus the severity of hepatic damage correlates well with the extent of covalent binding of metabolite(s) of radiolabelled bromobenzene. Pre-treatments which alter the magnitude of covalent binding of bromobenzene to tissue macromolecules in vivo cause parallel changes in the severity of hepatic lesions.

B - Structure activity relationship of halobenzenes

Structure activity relationship studies on halobenzenes have

shown a good correlation between the extent to which individual members of this group of compounds become covalently bound to liver proteins and the severity of hepatic lesions. In rats doses of 1 mmol/kg of radiolabelled chlorobenzene, bromobenzene, iodobenzene and O-dichlorobenzene cause liver necrosis and become covalently bound to a considerable extent to liver proteins whereas equimolar doses of fluorobenzene and p-dichlorobenzene do not cause liver necrosis and do not become covalently bound to liver proteins (Brodie et al, 1971; Reid, 1973; Reid and Krishna, 1973).

C - Dose-response relationships for bromobenzene

There is a threshold for the hepatotoxic effect of bromobenzene. Beyond this threshold dose bromobenzene becomes covalently bound to a considerable extent and causes liver necrosis. Covalent binding to liver proteins remains low until a dose between 1.2 and 2.15 mmol/kg is used. Above this critical dose the proportion of the dose that becomes covalently bound to liver proteins is nearly doubled and liver necrosis is manifested (Reid, 1973; Reid and Krishna, 1973).

Studies on the metabolism of bromobenzene have provided an answer for this threshold dose phenomenon. The collective data on the metabolism of bromobenzene (Brodie et al, 1971; Zampaglione et al, 1973; Jollow et al, 1974) show that bromobenzene is metabolized to a great extent to a cytotoxic reactive metabolite, 3,4-bromobenzene epoxide. This epoxide can be detoxified by undergoing non-enzymatic rearrangement to form p-bromophenol or becoming hydrated to a dihydrodiol derivative under the influence of

the enzyme epoxide hydratase or becoming conjugated with glutathione. The formation of the epoxide-glutathione conjugate can occur spontaneously or enzymatically catalyzed by glutathione S-epoxide transferase. The steady state concentration of the epoxide would depend on the balance between the rate of epoxide formation and the rates at which this epoxide is converted into the phenol, glutathione conjugate and the dihydrodiol. Covalent binding to cellular macromolecules of hepatic cells does not occur until liver glutathione stores of these cells are depleted (Gillette, 1973; Jollow et al, 1974). The level of glutathione would depend on the balance between the rate of glutathione synthesis and the rate at which glutathione is consumed in the formation of the bromobenzene glutathione conjugate. With high doses of bromobenzene the rate of formation of the bromobenzene-glutathione conjugate is sufficiently high to deplete liver glutathione stores and excess epoxide reacts covalently with cellular macromolecules. In contrast, in animals receiving either low doses of bromobenzene (below the threshold dose) or, alternatively, high doses of bromobenzene after pre-treatment with SKF 525-A, the rate of the bromobenzene epoxide formation is slow, the formation of the epoxide-glutathione conjugate is slow and liver levels of glutathione are never depleted. Consequently the covalent binding of the bromobenzene reactive metabolite to cellular macromolecules remains minimal and hepatotoxicity is almost completely prevented (Reid and Krishna, 1973).

2) The acetaminophen hepatotoxicity model

A - Correlation of changes in the extent of covalent binding

with changes in severity of toxicity

Acetaminophen (paracetamol) is an analgesic drug. High doses of acetaminophen cause centrilobular hepatic necrosis in man (Prescott, Wright, Roscoe and Brown, 1971), rats (Boyd and Bereczky, 1966; Mitchell, Jollow, Potter, Davis, Gillette and Brodie, 1973) and mice (Mitchell, Jollow, Potter, Davis, Gillette and Brodie, 1973). Studies on the mechanism of the hepatotoxic effect of acetaminophen have shown that administration of radiolabelled acetaminophen (300-750 mg/kg), in mice, causes necrosis of hepatic cells and that radioactivity from acetaminophen became covalently bound to liver proteins. Pre-treatment of animals with mixed function oxidase inhibitor, viz piperonylbutoxide or cobaltous chloride decreased both the severity of liver necrosis and the extent of covalent binding of radioactivity to liver proteins. In contrast, pre-treatment of the animals with phenobarbitone, a mixed function oxidase inducer, increased the severity of the necrosis and also increased the amount of covalently bound acetaminophen metabolites. Both covalent binding and hepatic necrosis were dose dependent. After administration of a non-toxic dose of radiolabelled acetaminophen (0.01 mg/kg) only insignificant amounts of covalently bound radioactivity were found in liver. Autoradiographic studies have shown preferential accumulation of covalently bound radioactivity, from radiolabelled acetaminophen, in the centrilobular hepatic cells. Thus the severity of hepatic lesions induced by acetaminophen correlate with the extent of covalent binding of acetaminophen metabolites to liver proteins. The extent of covalent binding to severely affected

hepatic cells is greater than that to apparently normal cells. Also covalent binding was greater to the drug metabolizing subcellular fraction, viz the microsomal fraction. Lesser amounts of covalently bound metabolites were found in nuclei and mitochondria (Jollow, Mitchell, Potter, Davis, Gillette and Brodie, 1973).

B - Metabolic pathway catalyzing the bioactivation of acetaminophen to an electrophilic metabolite

In vitro studies (Potter, Davis, Mitchell, Jollow, Gillette and Brodie, 1973) indicate that the covalent binding of acetaminophen to cellular macromolecules is catalyzed by a cytochrome P-450 dependent mixed function oxidase. Covalent binding of radioactivity, from radiolabelled acetaminophen, to protein of microsomal preparations required the presence of reduced nicotinamide adenine dinucleotide phosphate and oxygen and was inhibited by carbon monoxide. The acetaminophen reactive metabolite is thought to be an N-hydroxy derivative (Potter, Davis, Mitchell, Jollow, Gillette and Brodie, 1973). This metabolite is highly electrophilic. It forms conjugates with nucleophilic compounds such as glutathione, cysteine and N-acetyl-L-cysteine. The formation of these conjugates can occur spontaneously (non-enzymatically). However with glutathione the reaction may occur spontaneously or be catalyzed by a cytoplasmic enzyme glutathione S-transferase. Thus addition of glutathione, L-cysteine or N-acetyl-L-cysteine to microsomal preparations, in vitro inhibits the covalent binding of the acetaminophen metabolite to microsomal protein by providing alternative nucleophilic sites for binding the reactive metabolite. The decrease in covalent binding

to microsomal proteins paralleled an increase in the formation of conjugate between acetaminophen and the nucleophilic agent added (Rollins and Buckpitt, 1979). A method for isolation and quantitative determination of the glutathione, cysteine and N-acetyl-L-cysteine conjugates of acetaminophen by high pressure liquid chromatography has been described (Buckpitt, Rollins, Nelson, Franklin and Mitchell, 1977).

C- Dose-response relationship and detoxifying pathway for acetaminophen

The cytotoxic chemically reactive metabolite of acetaminophen is detoxified by reacting preferentially with glutathione. Covalent binding of this metabolite to hepatic macromolecules does not occur until the availability of glutathione is exhausted through conjugation with the metabolite. Consistent with this view is that no covalent binding of radiolabelled acetaminophen to liver protein and no necrosis affects hepatic cells unless the liver glutathione is severely depleted. The extent of covalent binding of radiolabelled acetaminophen to liver protein and the decrease in liver glutathione levels correlates very well with the severity of necrotic changes affecting the liver (Jollow, Mitchell, Potter, Davis, Gillette and Brodie, 1973; Mitchell, Jollow, Potter, Gillette and Brodie, 1973; Potter, Davis, Mitchell, Jollow, Gillette and Brodie, 1973). Pre-treatment of rats with cysteine, a precursor of glutathione, or with cysteamine or dimercaprol, which presumably offer alternative nucleophilic sites for reaction with the acetaminophen reactive metabolite, decrease the covalent binding of the acetaminophen metabolite

to liver protein and the severity of hepatic lesions (Mitchell, Jollow, Potter, Gillette and Brodie, 1973; Mitchell, Jollow, Gillette and Brodie, 1973). In contrast, pre-treatment of rats with diethylmaleate, a compound which depletes liver glutathione (Boyland and Chasseaud, 1970), increases the extent of covalent binding of acetaminophen to liver proteins and increases the severity of liver necrosis (Mitchell, Jollow, Potter, Gillette and Brodie 1973).

Acetaminophen displayed a threshold dose phenomenon similar to that described previously for bromobenzene. The critical dose for acetaminophen in mice is about 300 mg/kg. Necrosis does not occur in mouse liver, unless the mice are treated with a dose greater than 300 mg/kg (Mitchell, Jollow, Potter, Davis, Gillette and Brodie, 1973).

3) Pyrrolizidine alkaloids

Pyrrolizidine alkaloids represent a naturally occurring hazard to livestock as they are present in certain poisonous plants, including Ragwort (Senecio Jacobaea). The most important toxic effects caused by these alkaloids is their pneumotoxic and hepatotoxic actions (Hooper, 1978). These alkaloids appear to cause tissue damage by becoming converted to highly electrophilic pyrrolic metabolites which alkylate tissue macromolecules. In support of this view, it has been demonstrated that the hepatotoxic effect of these alkaloids correlates well with the amounts of pyrrolic metabolites found in livers of rats given the alkaloids. Pre-treatment of rats with SKF 525-A, an inhibitor of drug microsomal enzymes, or pre-feeding sucrose decreases both the hepatotoxicity and the concentration of liver pyrroles after retrosine administration.

On the other hand, pre-treatment of rats with phenobarbital, an inducer of microsomal enzymes, increased the susceptibility of female rats, but decreased the susceptibility of males to retrosine (Mattocks, 1972). Increasing or decreasing the concentration of liver glutathione by administration of cysteine or chloroethanol decreased or increased, respectively, the susceptibility of male rats to the hepatotoxic effect of retrosine. This indicates that the hepatotoxic metabolite of retrosine is electrophilic and therefore it was surprising to find that retrosine did not deplete liver glutathione in rats (White, 1976).

6.1.4 - Metabolic activation of foreign compounds to chemically reactive pneumotoxic metabolites

The hepatotoxic effect of many xenobiotic compounds is based on metabolic conversion of these chemically inert foreign compounds to chemically reactive intermediates which alkylate or arylate cellular macromolecules of hepatocytes producing cellular injury. The realisation that the lung possesses microsomal enzyme systems similar to those catalyzing the metabolic activation of hepatotoxic substances to chemically reactive metabolites has stimulated considerable interest in the possibility that chemically-induced pulmonary diseases might also be mediated by reactive metabolites. There is no doubt that the liver is quantitatively the most important organ involved in biochemical defence against foreign compounds. The lung is exposed to two sources of foreign compounds; those present in inspired air and those carried to the lung with blood stream. Furthermore the lung is the only vascular structure which receives all of the

cardiac output during each circulation.

6.1.4.1 - The presence of xenobiotic compound-metabolizing enzyme systems in the lung

The mammalian lung possesses enzyme systems capable of performing the following biotransformation reactions:

1) Mixed function oxidation

In vitro studies on rabbits (Gram, Litterst and Mimnaugh, 1974) have shown that the lung possesses a mixed function oxidase system(s) qualitatively similar to that of the liver. Mixed function oxidase activity was located in the microsomal fractions in both the lung and liver. Other subcellular fractions such as the nuclear and mitochondrial fractions exhibited only very low mixed function oxidase activity which was attributed to microsomal contamination. Quantitatively the overall activity of the mixed function oxidase (biphenyl hydroxylase) was about the same in the lung and liver. However, the cytochrome P-450, a component of the electron transfer chain of the enzyme system, content of liver microsomes was almost eight times that of the lung in rabbits.

Requirements for optimal activity of mixed function oxidase activity of the lung and liver microsomes of rabbits were essentially the same. Both systems required reduced nicotinamide adenine dinucleotide phosphate or reduced nicotinamide adenine dinucleotide phosphate-generating system and oxygen and were inhibited by carbon monoxide, cytochrome c and SKF 525-A (Bend, Hook, Easterling, Gram and Fouts, 1972; Gram et al, 1974). Mixed function oxidase activity of microsomal preparations from the lung were less than 3% of those

from the liver in man (measured with benz-pyrene, phenacetin and 7-ethoxycoumarin, as substrates (McManus, Boobis, Pacifici, Frempong, Brodie, Kahn, Whyte and Cavies, 1980). Examination of products of metabolism of polycyclic hydrocarbons benz (a) anthracene, benz (a) pyrene and 7-methyl benz (a) anthracene by microsomal preparations indicates that epoxides are formed from these compounds by cytochrome P-450 dependent mixed function oxidases of the lung and liver of rats. Microsomal preparations from the rat lung were at least as active as those from the rat liver in the metabolism of polycyclic hydrocarbons to chemically reactive epoxides (Grover, 1974; Grover, Hewer and Sims, 1974).

2) Conjugation reactions

A - Glucuronic acid conjugation

Glucuronic acid conjugation is an extremely important biotransformation reaction. Compounds undergoing this reaction include alcohols, phenols, carboxylic acids, amines, amides and thiols. This reaction proceeds in two steps. First the activation of glucuronic acid to form uridine diphosphate glucuronic acid, then the transfer of glucuronic acid from this activated nucleotide to an acceptor molecule to form glucuronide. The synthesis of the glucuronide is mediated by a microsomal enzyme, glucuronyl transferase (Baggot, 1977b).

Uridine dinucleotide phosphate-glucuronyl transferase activity in lung microsomal preparations was non-detectable (p-nitrophenol or phenolphthalein as substrates) or detectable in much lower amounts (O-aminophenol as substrate) than that in liver microsomal preparations from rabbits (Gram et al, 1974).

B - N-Acetylation

This reaction involves acetylation of amino groups of foreign compounds such as aromatic amines, sulfonamides and aromatic amino acids. The active acetylating agent is acetyl-CoA, which reacts with free amino groups on the drug to form an amide bond. The reaction is catalyzed by the microsomal enzyme N-acetyl transferase. N-Acetyl transferase activity towards para-amino benzoic acid in lung microsomal preparations was comparable to that of the liver. On the other hand, the activity in the lung when measured with sulfadiazine was much lower than that of the liver (Gram et al, 1974).

C - Methylation

Methyltransferases transfer methyl groups from S-adenosyl-methionine to a phenolic hydroxyl group, to a sulfydryl group or to various amino groups.

In vitro studies indicate that primary amines (e.g. desdimethyl-imipramine) are slowly methylated, whereas secondary amines (e.g. nortriptyline and desmethylimipramine) are rapidly methylated by an enzyme in the soluble fraction of rabbit lung (Dingell and Sanders, 1966). An enzyme catalyzing the transfer of the methyl group of S-adenosylmethionine to phenol has been demonstrated in microsomal preparations from lung and liver of rabbits and guinea pigs (Axelrod and Daly, 1968).

D - Glutathione conjugation

Glutathione S-transferases catalyze the conjugation of glutathione with a wide variety of foreign compounds and play an essential role in the detoxication of foreign compounds. The role of

glutathione S-transferases in biochemical defence and distribution of these enzymes in different tissues will be discussed in detail (see 6.1.5).

3) Epoxide hydrase

This reaction is catalyzed by epoxide hydrase enzyme. The distribution of this enzyme in different tissues and its role in detoxication of epoxides will be discussed (see 6.1.5.) This metabolic aspect of the lung and its possible implication in the aetiology of pulmonary diseases is inadequately investigated. There are only a few known examples of foreign chemicals which cause specific pneumotoxicity when administered by routes other than inhalation. Among these chemicals are the halobenzenes and furano compounds. The mechanism of action of these two groups of compounds towards the lung is discussed below.

6.1.4.2 - Examples of reactive metabolite-mediated pneumotoxicities

1) Pulmonary toxicity caused by halobenzenes

Halobenzenes cause hepatotoxicity by becoming converted to chemically reactive cytotoxic metabolites. This led Reid, Ilett, Glick and Krishna (1973), who observed that these compounds also caused lung lesions, to investigate the possibility that the halobenzene-pneumotoxicity is also mediated by a reactive metabolite. These investigators found that a single intreperitoneal dose of bromobenzene (4.85 mmol/kg), chlorobenzene (5.3 mmol/kg), naphthalene (2.75 mmol/kg) or O-dichlorobenzene (6.9 mmol/mg) caused severe pulmonary lesions in mice, whereas doses of up to 31 mmol/kg of P-dichlorobenzene

did not produce pathological changes in lung of mice. Rats were considerably more resistant to the pneumotoxic effect of bromobenzene than mice with some inter-individual variations. A high dose of bromobenzene (9.3 mmol/kg) induced pulmonary lesions, similar to those observed in mice, in less than 20% of treated rats.

Radioactivity from radiolabelled bromobenzene became covalently bound to proteins of many tissues. The highest concentration of covalently bound radioactivity was found in the liver, lung and kidney of treated mice. Only these organs showed necrotic changes (Reid et al, 1973).

Histopathological changes in lungs of halobenzene-treated mice were seen in the bronchiolar epithelium. The bronchiclar epithelial cells showed rounded appearance, pyknotic nuclei and increased acidophilia of the cytoplasm 24 to 36 hours after administration of bromobenzene. By 48 to 72 hours the bronchiolar epithelium showed complete coagulative necrosis. In contrast there was no alteration in alveolar morphology. Autoradiographic studies on tissue sections from the lung of mice receiving toxic doses of radio-labelled bromobenzene and chlorobenzene have shown that most covalently bound radioactivity in lung tissue was present in necrotic cells of the bronchial epithelium suggesting a causal relationship between the bronchiolar necrosis and the accumulation of covalently bound radioactivity (Reid et al, 1973). Thus the highest concentration of covalently bound metabolites occurred in affected organs (liver, lung and kidney) and within the lung severely affected cells (bronchial epithelium) showed preferential accumulation of covalently bound

metabolites. In view of the results of *in vitro* studies indicating that the enzyme system catalyzing the metabolic activation of bromobenzene is a cytochrome P-450 dependent mixed function oxidase, it has been attempted to use inducers (phenobarbitone) and inhibitors (piperonyl butoxide) of microsomal enzymes to study the correlation between the extent of covalent binding of bromobenzene and the severity of pulmonary lesions. These studies were not conclusive, being complicated by the fact that enzyme inducers and inhibitors may affect both toxifying as well as detoxifying pathways (Reid *et al*, 1973).

2) Pulmonary toxicity caused by furano compounds

The discovery that many furano compounds possess specific pulmonary toxic effects resulted from studies which had been conducted to characterize the pneumotoxic factor(s) of mouldy sweet potato causing lung lesions in cattle fed such a mouldy sweet potato. It has been known in the southern east states of the United States of America for many decades that feeding mouldy or rotted sweet potato (ipomoea batatas) causes clinical signs and pulmonary lesions characteristic of atypical interstitial pneumonia (Hansen, 1928; Monlux, Fitte, Kendrick and Dubuisson, 1953; Vickers, Carll, Brierer, Thomas and Valentine, 1960; Gibbons, 1962). A fungus Fusarium Solani was isolated from mouldy sweet potato producing the disease. The pulmonary disease was experimentally produced by oral administration of homogenized sweet potato cultures infested with this fungus. Clinical signs in affected cattle were severe respiratory distress, with a rapid respiratory rate. Froth around the mouth, extension of the head and laboured breathing were observed before death.

Pathological changes were confined to the lungs which showed marked oedema, alveolar and interstitial emphysema, alveolar epithelial hyperplasia, hyaline membranes and congestion (Peckham, Mitchell, Jones and Doupnik, 1972).

Characterization of the pulmonary toxic factors in mould-damaged sweet potatoes has recently been accomplished. It was shown that this pneumotoxic factor comprises a group of at least four closely related furanoterpenoids: 4-ipomeanol, 1-ipomeanol, ipomeanine and 1,4-ipomeadiol. All these compounds were found to be acutely toxic to the lungs of experimental animals. Additionally, it was shown that mice initially surviving near-lethal doses of these toxic compounds, particularly 1-ipomeanol and 1,4-ipomeadiol, may show evidence of nephrotoxicity within one to three days (Boyd, Burka, Harris and Wilson, 1973).

A - Correlation of changes in covalent binding with changes in severity of lesions

Preliminary studies (Boyd, Burka and Wilson, 1975) indicated that the metabolism of 4-ipomeanol involves the formation of reactive metabolite(s). Radiolabelled 4-ipomeanol became covalently bound to many tissues after intraperitoneal administration in rats. Lung tissue showed the highest concentration of covalently bound metabolites, suggesting a causal relationship between covalent binding of 4-ipomeanol metabolites to lung tissue and the 4-ipomeanol-induced pulmonary damage.

In vitro studies (Boyd, Burka, Wilson and Sasame, 1978) on the enzyme system mediating the covalent binding of 4-ipomeanol

to cellular macromolecules revealed that this enzyme system is a cytochrome P-450 dependent mixed function oxidase. Consistent with this view, pre-treatment of rats with inhibitors of mixed function oxidases (pyrazole, piperonyl butoxide and cobaltous chloride) before administration of radiolabelled 4-ipomeanol decreased both the toxicity and the extent of covalent binding of 4-ipomeanol metabolites to lung proteins in rats. In contrast pre-treatment of rats with mixed function oxidase inducers (phenobarbital and 3-methylcholanthrene) before administration of radiolabelled 4-ipomeanol increased both the toxicity and extent of covalent binding of 4-ipomeanol metabolites to lung proteins (Boyd and Burka, 1978).

In vitro studies (Boyd, Burka, Wilson and Sasame, 1978) on the nature of the reactive metabolite of 4-ipomeanol showed that the metabolite is electrophilic. Addition of the nucleophilic substance glutathione to microsomal preparations containing radiolabelled 4-ipomeanol strongly prevented covalent binding of 4-ipomeanol metabolite(s) to microsomal proteins. Furthermore addition of the cytosol fraction of the rat lung (contains glutathione S-transferase) with or without glutathione inhibited covalent binding to a greater extent than did glutathione alone (Boyd, Burka, Wilson and Sasame, 1978). These findings indicate that the reactive metabolite of 4-ipomeanol can be detoxified by conjugation with glutathione. The importance of glutathione in the detoxication of the 4-ipomeanol reactive metabolite was confirmed by in vivo studies (Boyd and Burka, 1978). Pre-treatment of rats with diethylmaleate to deplete glutathione increases both the pulmonary toxic effect of 4-ipomeanol

and the extent of covalent binding of chemically reactive metabolite(s) of 4-ipomeanol to lung proteins.

Thus the collective data on 4-ipomeanol indicate that the extent of covalent binding of 4-ipomeanol metabolite(s) correlates well with the severity of lung lesions (Boyd, 1976; Boyd and Burka, 1978; Boyd, Burka, Wilson and Sasame, 1978).

B - The Clara cell as a primary target for reactive metabolite forming pneumotoxic substances

Clara cells are tall non-ciliated respiratory epithelial cells. These cells are most frequent in the epithelial lining of bronchioles. This cell shows an abundance of smooth endoplasmic reticulum in its cytoplasm (Jeffrey and Reid, 1975), suggesting that this type of cell is active in metabolizing drugs and foreign compounds.

Boyd and Burka (1978) cited results of unpublished work indicating that Clara cell necrosis is a consistent and highly reproducible feature of 4-ipomeanol pneumotoxicity in all animal species investigated. Pulmonary oedema is not a consistent feature; in some species, such as the mouse, extensive Clara cell necrosis produced by 4-ipomeanol or other furano compounds may frequently be accompanied by little or no pulmonary oedema. Another furano compound, 3-methylfuran, caused specific necrosis of Clara cells (Boyd, Statham, Franklin and Mitchell, 1978). Thus the Clara cell appears to be a primary target for 4-ipomeanol (Boyd, 1977; Boyd and Burka, 1978) and 3-methylfuran (Boyd, Statham, Franklin and Mitchell, 1978).

It has been suggested that the Clara cell is the site of

cytochrome P-450 dependent mixed function oxidases in the lung. This would make this type of cell more susceptible than other cell types to reactive metabolite forming pneumotoxic substances where metabolic activation is mediated by a cytochrome P-450 dependent mechanism (Boyd, 1977). Consistent with this theory is the finding that covalently bound radioactivity from 4-ipomeanol and 3-methylfuran was localized in this cellular type as shown by autoradiographic studies. These autoradiographic studies were performed on tissue sections from the lungs after administration of toxic doses of radiolabelled 4-ipomeanol to rats, guinea pigs and hamsters or radiolabelled 3-methylfuran to mice. In the three animal species tested, the Clara cells, which were necrotic, showed accumulation of black photographic granules, indicating accumulation of covalently bound radioactivity, whereas the adjacent ciliated bronchiolar cells, and other cellular types of the lung, were not necrotic and did not show evidence of radioactivity. This specific alkylation of Clara cells was shown to be dependent on the metabolism of these furano compounds. Inhibitors of mixed function oxidases (piperonyl butoxide) prevented Clara cell necrosis and accumulation of radioactivity in these cells after administration of radiolabelled 4-ipomeanol or radiolabelled 3-methylfuran (Boyd, 1977; Boyd, Statham, Franklin and Mitchell, 1978).

The Clara cell is one of the cellular types forming the epithelial lining of small airways. The relative proportion of Clara cells to other cellular types increases as airway size decreases (Jeffrey and Reid, 1975). In parallel with this, the

sensitivity of airways to the toxic effect of 4-ipomeanol and 3-methylfuran increases as the airway size decreases (Boyd, 1977; Boyd, Statham, Franklin and Mitchell, 1978).

Thus the vulnerability of an airway to the toxic effect of pneumotoxic furano compounds appears to be determined by the relative number of metabolically active Clara cells present in the epithelial lining of the airway. With small doses of 4-ipomeanol or 3-methyfuran both bronchiolar necrosis and covalent binding of radioactivity were restricted to the smallest bronchioles; with increasingly larger doses larger airways became progressively involved (Boyd, 1977; Boyd, Statham, Franklin and Mitchell, 1978).

C - Organ specificity of 4-ipomeanol

Although the enzyme system responsible for the toxicity of 4-ipomeanol is present in both the liver and lung, 4-ipomeanol possesses pneumotoxic but no hepatotoxic effect. The possibility that this specific pneumotoxic effect is due to a higher rate of reactive metabolite formation in the lung than in the liver was investigated by Boyd, Burka, Wilson and Sasame (1978). These investigators incubated microsomal preparations from the lung and liver of rats with different concentrations of radiolabelled 4-ipomeanol and the rate of covalent binding of 4-ipomeanol metabolite(s) to microsomal protein was measured. The Michaelis constant (the concentration of 4-ipomeanol required to obtain half the maximal rate of microsomal alkylation) for the lung microsomal system was more than ten-fold lower than for the hepatic microsomal system. The

maximal rates of lung and liver microsomal alkylation were about equal magnitude (0.45 and 0.51 nmol bound 4-ipomeanol/mg microsomal protein/minute for the lung and liver respectively) when covalent binding of 4-ipomeanol to microsomal proteins was calculated in terms of covalent binding per mg of microsomal protein. However, when the maximal velocity values were calculated per nanomole of cytochrome P-450, the maximal velocity value appeared much higher for lung microsomes (4.85 and 0.48 nmol bound 4-ipomeanol/nmol P-450/minute for the lung and liver respectively) because of the much lower levels of cytochrome P-450 in lung.

D - Dose-response relationships of 4-ipomeanol

There is a threshold dose for 4-ipomeanol in rats (about 20 mg/kg, intraperitoneally) above which there is a marked increase in the severity of pulmonary oedema. Fatalities start to appear above this threshold dose (Boyd and Burka, 1978).

E - Structure-activity relationship studies on 4-ipomeanol analogues

Analogues of 4-ipomeanol in which the furan moiety is replaced with either a phenyl or a methyl substituent did not cause enzymatic-dependent alkylation of microsomal proteins when these analogues were incubated with microsomal preparations. Thus it has been proposed that the furan moiety of the 4-ipomeanol molecule is required for enzymatic activation of 4-ipomeanol to an alkylating agent. Furthermore it has been proposed that the reactive metabolite of 4-ipomeanol is probably an epoxide. This hypothesis is based on analogy with toxic furano compounds (Boyd, Burka, Wilson and Sasame,

1978).

6.1.5 - Metabolic pathways involved in the generation and elimination of chemically reactive metabolites of foreign compounds

1) Epoxidation

A - The mechanism of formation of epoxides by mixed function oxidases

Epoxides are three-membered cyclic ethers. Epoxides resulting from the epoxidation of one of the double bonds of an aromatic nucleus are called arene oxides. The name alkene oxide has been suggested for epoxides resulting from the epoxidation of an olefinic double bond (Oesch, 1972). Epoxides can be formed by the action of microsomal mixed function oxidases on molecules having aromatic rings or alkene bonds. The formation of an epoxide is the first step in the oxidation process where mixed function oxidases are involved (Hamilton, 1964; Holtzman, Gillette and Milne, 1967; Jerina, Daly, Witkop, Zaltzman-Nirenberg and Udenfriend, 1970; Grover, 1974). When naphthalene was incubated with microsomal preparations in the presence of reduced nicotinamide adenine dinucleotide phosphate under an atmosphere of oxygen-18, the diol formed was shown by mass spectrometry to contain only one atom of oxygen-18. These observations are compatible with the intermediate formation of 1,2-dihydro-1,2-epoxynaphthalene, and that the oxygen atom of this epoxide is derived from atmospheric oxygen. Microsomal mixed function oxidase enzymes converted naphthalene to 1,2-dihydro-1,2-epoxy naphthalene, which in turn reacts with either water to form 1,2-dihydronaphthalene-1,2-diol

or glutathione to form S-(1,2-dihydro-2-hydroxyl-1-naphthyl) glutathione (Holtzman, Gillette and Milne, 1967). Isolation of the naphthalene epoxide intermediate has recently been achieved. The problem of instability of the epoxide was overcome by incubating microsomal preparations at low temperature (30°C) for a short time (three minutes) or by trapping the epoxide in a large pool of non-labelled epoxide. Furthermore in the presence of rat liver microsomal preparations 1,2-naphthalene oxide was converted to all major naphthalene metabolites; these are 1-naphthol, the diol and the naphthalene-glutathione conjugate. The formation of the 1-naphthol was shown to occur by non-enzymatic rearrangement of the naphthalene epoxide whereas the formation of the diol from the epoxide and the conjugation of the epoxide with glutathione were catalyzed by different epoxide metabolizing enzymes. Based on these findings it has been proposed that the 1,2-naphthalene oxide is an obligatory intermediate for all the naphthalene metabolites (Jerina, Daly, Witkop, Zaltzman-Nirenberg and Udenfriend, 1970).

It has been proposed that the formation of arene oxides from aromatic hydrocarbons by the action of mono-oxygenases is probably a general phenomenon (Oesch, 1972). The isolation of these epoxides is difficult since they are very unstable. Observation of ultimate metabolites, such as dihydrodiols, phenols and glutathione conjugates, is therefore a good indication of the formation of an intermediate epoxide (Oesch, 1972).

B - Examples of metabolic epoxidation and toxicological implications of epoxide

Boyland (1950) hypothesised that epoxidation is an initial

step in the metabolism of hydrocarbons. However because of the highly reactive nature of epoxides, the existence of epoxides was not proved until the relatively stable epoxides of some insecticidal chlorinated hydrocarbons, initially heptachlor, were isolated. These epoxides are poor substrates for epoxide hydrase possibly contributing to their stability (Oesch, Kaubisch, Jerina and Daly, 1971). The epoxide of the insecticide aldrin, is dieldrin, which is sufficiently stable to cause cumulation problems in food chains. Aldrin requires to be metabolized to dieldrin for insecticidal activity (Brooks, Harrison and Cox, 1963). Male rats produced more epoxide from aldrin and heptachlor than females; in parallel with this female rats are more resistant than males to the toxic effects of these compounds suggesting that the toxic effects of these compounds are mediated through their epoxides (Radomski and Davidow, 1953; Wong and Terriere, 1965).

Epoxides are the chemically reactive metabolites responsible for the toxic effect of many carcinogens. Aflatoxins, a group of naturally occurring carcinogenic mycotoxins, include four compounds aflatoxin B_1, B_2, G_1 and G_2. Aflatoxin B_1 possesses the greatest carcinogenic activity and is probably the most potent liver carcinogen known for the rat (Wogan and Newberne, 1967; Carnaghan, 1967). Also it is capable of inducing kidney tumours in rats (Butler, Greenblatt and Lijinsky, 1969) and colon carcinoma in vitamin -A deficient rats (Newberne and Rogers, 1973). Early studies on the mechanism of the carcinogenic action produced by aflatoxin B_1 indicated that aflatoxin B_1 can alkylate nucleic acids <u>in vivo</u>.

Lijinsky, Lee and Gallagher (1970) demonstrated that radicactivity from radiolabelled aflatoxin B_1 became covalently bound to liver cellular macromolecules of rats after administration of tritiated aflatoxin. The parent compound aflatoxin is chemically unreactive and does not alkylate nucleic acids in vitro. Thus the in vivo metabolism is essential for covalent binding of aflatoxin B_1 to cellular macromolecules. Results of recent work indicate that the aflatoxin B_1 reactive metabolite is an epoxide. A simple method of studying highly reactive metabolites is to identify the covalently bound forms of the compound. This strategy was adopted with aflatoxin B_1. Acid hydrolysis of the aflatoxin B_1-ribonucleic acid conjugate cleaves the aflatoxin B_1 moiety to 2,3-dihydro-2,3-dihydroxy aflatoxin B_1, suggesting that the aflatoxin B_1-ribonucleic acid conjugate is formed by the covalent binding of the electrophilic metabolite 2,3-epoxy-2,3-dihydraflatoxin B_1 to nucleophilic sites on the ribonucleic acid molecule (Swenson, Miller and Miller, 1973).

Examination of products of metabolism of the suspected carcinogens benz (a) anthracene, benzo (a) pyrene and 7-methylbenz (a) anthracene indicate that epoxides are formed from these compounds by mixed function oxidases of the liver and lung of rats. Metabolites formed from these polycyclic hydrocarbons were mainly ring hydroxylated derivatives, phenols and dihydrodiols. These metabolites arise from epoxide intermediates (Grover, Hewer and Sims, 1974).

C - Fate of epoxides

(a) Spontaneous rearrangement

Epoxides undergo spontaneous non-enzymatic rearrangement to

the corresponding phenols. This has been observed with many arene oxides (Jerina, Daly and Witkop, 1968; Jerina, Kaubisch and Daly, 1971; Boyd, Daly and Jerina, 1972).

(b) Conjugation with glutathione

The reaction between epoxides and glutathione occurs spontaneously, but also an enzyme glutathione S-epoxide transferase can catalyze this reaction. Glutathione S-epoxide transferase belongs to a group of enzymes known as glutathione S-transferases. These enzymes catalyze the conjugation of glutathione with a wide variety of cytotoxic electrophilic compounds and are present in the microsomal supernatant fractions (cytosol or soluble cytoplasm) (Boyland and Chasseaud, 1969; Wood, 1970; James, Fouts and Bend, 1976). Thus they play an important role in the elimination of many cellular alkylating agents. Using ion exchange chromatography, it has been possible to isolate six different transferases from rat liver. According to the order of their elution from carboxymethylcellulose columns, the rat liver transferases are designated E, D, C, B, A and AA (Habig, Pabst and Jacoby, 1974). Transferase B is called ligandin (Habig, Pabst, Fleischner, Gatmaitan, Arias and Jacoby, 1974). The glutathione S-transferases have a broad and overlapping substrate specificity (Habig, Pabst and Jacoby, 1974; Nemoto, Galboin, Habig, Ketley and Jacoby, 1975). It has been shown that rat liver glutathione S-epoxide transferase is inducible by administration of phenobarbital or 3-methylcholanthrene (Mukhtar and Bresnick, 1976).

Formation of glutathione-foreign compound conjugate is the first step towards the formation of mercapturic acids. The glutathione

moiety of the molecule loses glutamate and glycine and undergoes N-acetylation of the remaining cysteine residue to form premercapturic acids. In acidic medium these premercapturic acids easily dehydrate to form mercapturic acids (Boyland, Ramsay and Sims, 1961; Chasseaud, 1976).

(c) Detoxication of epoxides by epoxide hydrase

The epoxide hydrase enzyme plays an important role in further metabolism of epoxides. This enzyme converts chemically reactive epoxides to much less reactive diols. Substrates for this enzyme include epoxides of a great variety of widely differing chemical compounds (Oesch, 1972). Epoxide hydrase is a microsomal enzyme and might be used as a marker enzyme for microsomal membranes (Oesch and Daly, 1971; Oesch, 1972; James et al, 1976).

Hepatic epoxide hydratase is inducible. Supplementation of rat diet with dietary antioxidants, butylated hydroxytoluene, butylated hydroxyanisole or ethoxyquin resulted in increased liver epoxide hydratase activity (Kahl and Wulff, 1979). This probably explains the protective effect of these compounds against chemical carcinogenesis (Wattenberg, 1972; Ulland, Weisburger and Yamamoto, 1973; Wattenberg, Loub, Lamb and Speier, 1976; Slaga and Bracken, 1977).

(d) Distribution of epoxide metabolizing enzymes in different organs as an explanation for organ specificity in toxicity

James et al (1976) investigated the distribution of glutathione S-epoxide transferase and epoxide hydrase activities in different

organs of the rabbit. The enzyme glutathione S-epoxide transferase was determined in the microsomal supernatant fraction obtained from different organs (styrene oxide as a substrate). Soluble fraction from the liver had the highest specific activity. Specific activities of soluble fractions from the kidney and lung were about 25 and 20% of the specific activity of the liver respectively. Intestinal mucosa showed the lowest activity and was about 15% of the liver. Epoxide hydrase activity was determined in microsomal fractions from different organs (styrene oxide as a substrate). Liver microsomes showed the highest specific activity, followed by intestinal mucosa microsomes. Lung microsomes showed the lowest epoxide hydrase activity (about 5% of the liver).

Glutathione S-aryltransferase activity in the cytosol fraction of the liver of the rabbit was about five times higher than that in the cytosol fraction of the lung, when 2,3-dichloronitrobenzene was used as a substrate (Gram et al, 1974).

Glutathione S-epoxide transferase activity in the human lung is about 23% of that of the liver, while rat lung microsomes possess 14% of the activity of the liver. Epoxide hydratase activity in human and rat lung is about 11% of that of the liver (styrene oxide as a substrate) (McManus, Boobis, Pacifici, Frempong, Brodie, Kahn, Whyte and Davies, 1980).

The comparatively low ability of the lung to metabolize epoxides particularly by the epoxide hydrase pathway may be a significant factor in the susceptibility of the lung towards polycyclic hydrocarbon carcinogens (James et al, 1976).

2) Other mechanisms

N-hydroxylation by microsomal mixed function oxidases is thought to be an important step in the mechanism of bioactivation of carcinogenic aminoazo dyes and aromatic amines. The proposed mechanism of bioactivation of aminoazo dyes such as N,N-dimethylaminoazobenzene in the liver is thought to involve N-hydroxylation mediated by endoplasmic reticulum mixed function oxidases followed by sulfate esterification mediated by cytoplasmic sulfotransferases (King and Phillips, 1968; De Baum, Miller and Miller, 1970; Weisburger, Yamamoto, Williams, Grantham, Natsushima and Weisburger, 1972; Heidelberger, 1975; Miller and Miller, 1975). A similar mechanism has been suggested for aromatic amines such as N-2-fluorenylacetamide; also this compound may be activated by esterification to an acetate (Miller, 1970; Bartsh, Dworkin, Miller and Miller, 1973; King, 1974).

N-oxidation of secondary and tertiary N-alkylarylamines can be catalyzed by a flavoprotein-requiring, non-cytochrome P-450, mixed function oxidase (Masters and Ziegler, 1971), whereas N-oxidation of primary arylamines and N-acetylarylamines can be catalyzed by a cytochrome P-450 dependent mixed function oxidase (Uehleke, Schnitzer and Hulmer, 1970; Thorgeirsson, Jollow, Sasame, Green and Mitchell, 1973).

Metabolic activation of carcinogenic dialkylnitrosamines involves cytochrome P-450 mediated N-demethylation of the parent compound to monoalkylnitrosamines, which undergo spontaneous rearrangement to unknown ultimate carcinogens (Magee and Barnes, 1967).

Glutathione plays an important role in the detoxication of chemically reactive metabolites. Because of their broad substrate specificities, glutathione S-transferases can catalyze the conjugation of electrophilic metabolites of a wide spectrum of chemically different compounds with glutathione, thus protecting nucleophilic sites on cellular macromolecules against becoming alkylated by these reactive metabolites (Smith, Ohi and Litwak, 1977).

Bioactivation to cytotoxic metabolites is the mechanism of action of many anti-cancer drugs. These drugs are designed so that they can be bioactivated to cytotoxic metabolites only by cancer cells. Therefore they cause selective destruction of these cells. These anti-cancer drugs can be metabolically activated to alkylating metabolites by reduction, hydrolysis and mixed function oxidation. These mechanisms are reviewed by Connors (1976). Thus the phenomenon of metabolic activation to chemically reactive cytotoxic metabolites can advantageously be exploited to destroy cancer cells.

6.2 EXPERIMENTAL

6.2.1 - Materials

Indole, 3-methylindole, Ehrlich reagent, florisil, L-cysteine, N-acetyl-L-cysteine and glutathione were obtained from Sigma Chemical Co. Ltd., London. Nicotinamide adenine dinucleotide phosphate, reduced nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and cytochrome c (horse heart) were obtained from Boehringer Corporation, Mannheim, Germany. SKF 525-A was a gift from Smith, Kline and French, Philadelphia, Pa. The

liquid scintillation cocktail, Unisolve, was obtained from Koch-Light Ltd., Colnbrook, Bucks, England. Other chemicals were commercially available and were of analytical grade. L-(G-^{3}H) tryptophan and L-(methylene ^{14}C) tryptophan were obtained from Radiochemical Centre, Amersham, England.

6.2.2 - Preparation of radiolabelled indole, 3-methylindole and 3-methyloxindole

There is no method reported for the preparation of radiolabelled indole, 3-methylindole and 3-methyloxindole, none of which is commercially available. Results presented in Chapter 2 show that bovine ruminal fluid converts L-tryptophan to 3-methylindole, indole and other metabolites. N-hexane selectively extracted 3-methylindole and indole from incubation mixtures after incubation of radiolabelled L-tryptophan with ruminal fluid. In this present method radiolabelled L-tryptophan was converted to 3-methylindole and other indolic metabolites by ruminal fluid. 3-Methylindole was extracted by n-hexane and isolated on florisil columns.

Preparation of (G-^{3}H) indole and (G-^{3}H) 3-methylindole

Substrate

L-(G-^{3}H) tryptophan was stated to be 95-98% radiochemically pure as determined by thin layer chromatography and dilution analysis. The specific activity was 15 mCi/mg or 3.1 Ci/mmol.

Procedure of microbiological synthesis

Tritiated L-tryptophan (5 mCi) together with a carrier amount of L-tryptophan (2 mg) was added to 20 ml ruminal fluid in a conical flask (50 ml capacity) and incubated under anaerobic conditions (carbon

dioxide) at 37°C in a shaking water bath, for 24 hours. The ruminal fluid was extracted twice with 2 x 50 ml of redistilled n-hexane. The combined hexane fraction was washed with 5 ml 1N NaOH followed by 5 ml 1N HCl. The hexane fraction was dried with anhydrous sodium sulphate and filtered. The combined hexane fraction was evaporated under vacuum to a volume of about 5 ml. A solution of indole and 3-methylindole (500 µg/ml, in hexane) was added until the concentration of indole and 3-methylindole reached about 50 µg/ml.

33 g of florisil were activated at 110°C in an oven, allowed to cool to room temperature in a desiccator, 1 ml distilled water was added and vigorously shaken for 30 minutes. 3 g were taken in a glass column (0.3 x 50 cm). A small amount of anhydrous, hexane-washed sodium sulphate was added at the top of the column. The column was washed with 15 ml of 5% (v/v) ether in hexane followed by 25 ml hexane, and then dried (suction applied to the tip of the column). The hexane extract from the incubation mixture was applied and eluted with 0.5% ether in hexane. The first 40 ml were discarded, then 0.5 ml fractions were collected. The concentration of indole and 3-methylindole was measured in each fraction and fractions containing only indole or 3-methylindole were pooled and radiochemical purity of indole and 3-methylindole was determined.

Fractions containing both indole and 3-methylindole were pooled and concentrated to about 5 ml (reduced pressure) and the column procedure was repeated using another florisil column, to isolate further amounts of 3-methylindole and indole. Indole and 3-methylindole determinations were made using gas liquid chromatography as described

before (Chapter 2).

Determination of the radiochemical purity of tritiated indole and 3-methylindole

The radiochemical purity of radiolabelled indole and 3-methylindole was determined using thin layer chromatography on silica gel plates (100 μ thick). 5 μl of the solution of tritiated compound were spotted on thin layer plate together with an authentic sample of the compound in n-hexane (1 mg/ml). After development the 3-methylindole spot was visualized with Ehrlich reagent spray, and scraped into a scintillation vial. The plate was divided into four equal portions and each portion scraped into a scintillation vial. 10 ml of the liquid scintillator unisolve 1 were added, and radioactivity was measured using liquid scintillation counting. Details of the chromatographic procedure were as described previously (Chapter 2).

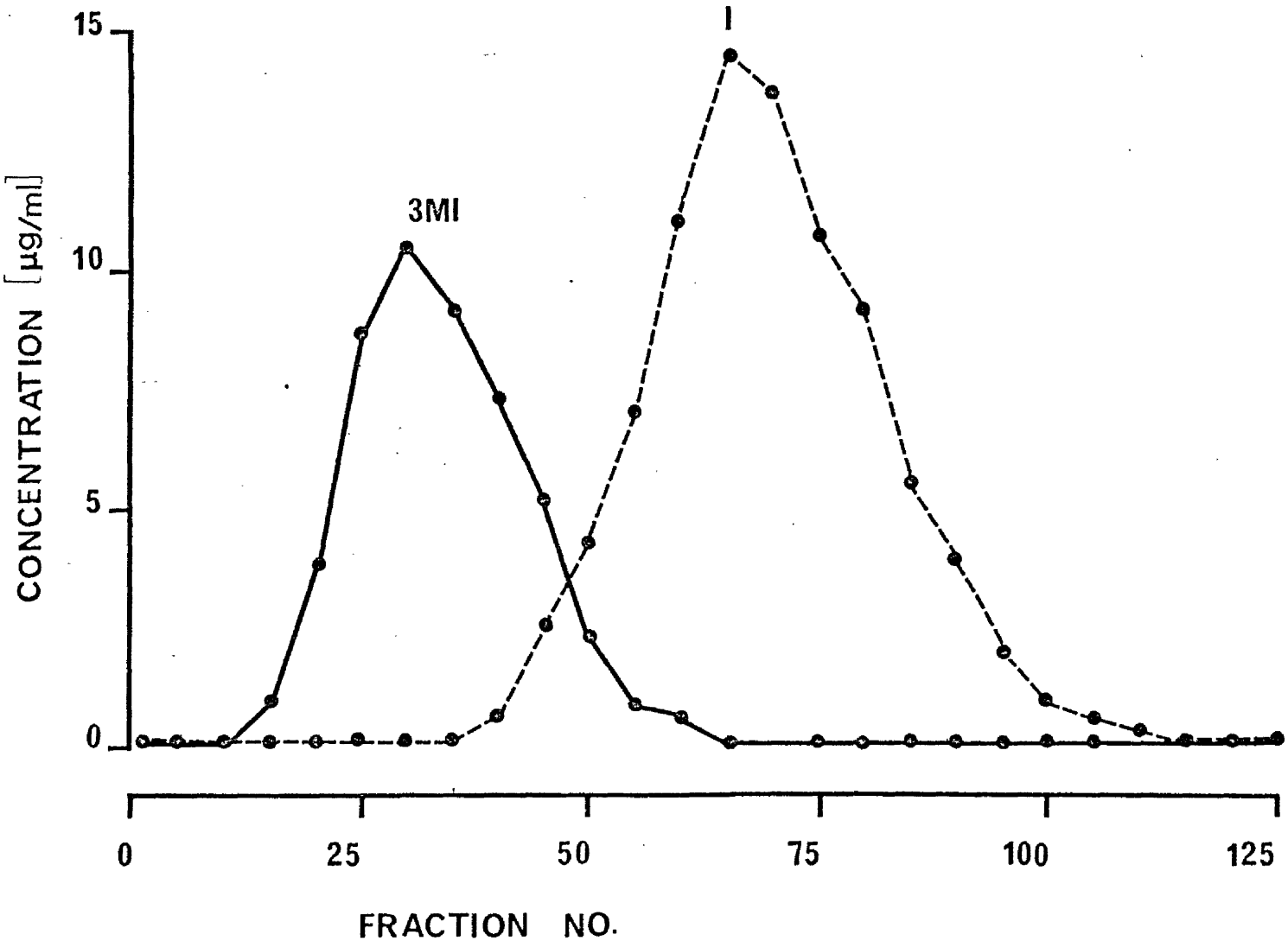
Results

The elution pattern of indole and 3-methylindole was as shown in Fig. 6.1. Resolution of indole from 3-methylindole was incomplete. 35 fractions contained 3-methylindole only followed by 30 fractions containing a mixture of indole and 3-methylindole, then 50 fractions containing indole. Repeating the column procedure for three times, it was possible to collect 800 μCi of tritiated 3-methylindole.

Only 20 μCi of indole were collected, the remainder of indole being discarded.

Radiochemical purities of tritiated indole and 3-methylindole

Fig. 6.1 Chromatographic separation of indole (I) and 3-methylindole (3MI) on a florisil column. Elution solvent was diethylether in hexane (0.5%, v/v). The first 40 ml were discarded and fractions of 0.5 ml collected.


15
10
5
0
CONCENTRATION [µg/ml]
3MI
I
0
25
50
75
100
125
FRACTION NO.

were as follows:

Solvent	Radiochemical purity (%) Indole	3-Methylincole
2-propanol-ammonia-water (20:1:2, v/v/v)	96.7	99.1
Cyclohexane-chloroform-diethylamine (4:5:1, v/v/v)	95.8	98.9
Di-isopropyl ether	90.5	99.47

Tritiated 3-methylindole was stored in a solution in redistilled n-hexane (20 mg/ml, 131 µCi/mmol) in a graduated, glass stoppered test tube at room temperature. Tritiated indole was similarly stored at a concentration of 1 mg/ml and 117 µCi/mmol specific activity.

Preparation of (methyl ^{14}C) 3-methylindole

Substrate

The specific activity of L-(methylene ^{14}C) tryptophan was 56 mCi/mmol or 272 µCi/mg and was stated to be 97% radiochemical pure as determined by high performance liquid chromatography.

Procedures

L-(methylene ^{14}C) tryptophan (50 µCi) together with a carrier amount of L-tryptophan (10 mg in 1 ml 0.1N NaOH) was added to 20 ml ruminal fluid in a conical flask (50 ml capacity) and incubated under anaerobic conditions (carbon dioxide) at 37^{o}C in a shaking water bath, for 24 hours.

Extraction, isolation, purification and radiochemical purity determination of ^{14}C labelled 3-methylindole were conducted as

described for tritiated 3-methylindole.

Results

A total of 20 µCi of ^{14}C labelled 3-methylindole was collected. The radiochemical purity of ^{14}C labelled 3-methylindole in three solvent systems was as follows:

Cyclohexane-chloroform-diethylamine (20:1:2, v/v/v)	99.8%
Propanol-ammonia-water (20:1:2, v/v/v)	99.5%
Di-isopropyl ether	99.3%

(Methyl ^{14}C) 3-methylindole was stored as a solution in n-hexane at a concentration of 1 mg/ml and specific activity of 131 µCi/mmol.

Preparation of tritiated 3-methyloxindole

3-Methyloxindole was prepared from 3-methylindole according to the method of Hinman and Bauman (1964)as follows:

To a solution of 3.28 mg of tritiated 3-methylindole (specific activity 131 µCi/mmol) in 163 µl of 95% t-butyl alcohol (in a 2 ml capacity test tube) were added 4.45 mg of N-bromosuccinimide. The reaction mixture was kept under nitrogen and at a temperature of 20^{o}C in a shaking water bath. After 2.5 hours the mixture was extracted with 1 ml ethyl acetate. The extract was dried over sodium sulphate. The concentration of 3-methyloxindole was measured using gas-liquid chromatography, as described in Chapter 3, and it was

found to be 600 µg/ml. 3-Methyloxindole was purified on silica gel thin layer plates.

Five (10 µl) aliquots of the ethylacetate extract were applied to the plates. After developing the plate in di-isopropyl ether, the solvent front was marked and the developed length divided into ten equal sections. The silica gel in each section was scraped into a test tube. Elution was done by adding 5 ml di-ethyl ether to the contents of each test tube. Each fraction was examined for the presence of 3-methyloxindole and 3-methylindole (gas-liquid chromatography). Fractions containing 3-methyloxindole only were saved.

About 500 µg of pure 3-methyloxindole were recovered in ether.

Preparation of solutions of indolic compounds for in vitro studies

The required amount of the solution of the labelled compound was accurately measured. The organic solvent was evaporated (vacuum, at 40°C). The indolic compound was redissolved in 1% Cremophor-EL in phosphate buffer pH 7.4. Aliquots were taken for determination of the concentration of the indolic compound by liquid scintillation counting or gas-liquid chromatography. More of the solvent was added to make the required concentration of indolic compound. Solutions of indolic compounds in Cremophor-EL buffer were freshly prepared before each experiment.

6.2.3 - Preparation of the microsomal and cytosol fractions from the bovine lung

Animals

Three 12 month old Ayrshire or Hereford-cross breed calves were used, unless otherwise indicated. Animals were stunned, using a captive bolt pistol, pithed with a light cane, and immediately exsanguinated by jugular sections. Pieces of lung tissue were removed and placed in a chilled preparation medium of 0.154M KCl and 0.05M Tris-HCl buffer.

Homogenization

All steps were carried out at 0-4^{o}C. Freshly removed tissue was chopped with a pair of scissors and excess connective tissue was removed.

The bovine lung is difficult to homogenize because of its fibrous nature. A blender type homogenizer was found to be preferable to a Potter-type glass-glass pestle homogenizer.

The chopped tissue (50 g) was homogenized in four volumes (200 ml) of ice-cold preparation medium of 0.154M KCl and 0.05M Tris-HCl buffer, pH 7.4 in an Atomix blender (M.S.E. Ltd., Manor Royal, Crawley, Sussex) for two minutes. The mixture was strained through gauze to remove fragments of connective tissue.

Ultracentrifugation

A Beckman L2-65B ultracentrifuge was used with type 50.1 rotor. The homogenized material was centrifuged at 16,000 xg for 20 minutes to remove cellular debris, nuclei and mitochondria. The 16,000 xg supernatant was carefully decanted into ultracentrifuge

tubes and centrifuged at 105,000 xg for 60 minutes.

The supernatant was saved for preparation of the cytosol fraction. For preparation of washed microsomes, microsomal pellets were rehomogenized in phosphate buffer (glass-glass pestle type homogenizer) pH 7.4 and recentrifuged at 105,000 xg for 60 minutes. Supernatants were discarded and microsomal pellets were saved for experimental work.

Mixed microsomes from three to five animals were used in each experiment.

Preparation of boiled microsomes

The microsomal suspension in a 10 ml capacity graduated test tube was placed in a boiling water bath for 15 minutes, with occasional shaking. This resulted in coagulation of microsomal proteins which tended to form large lumps. Boiled microsomes were allowed to cool, rehomogenized and the volume corrected for loss of water due to evaporation during boiling.

Preparation of the cytosol fraction from the bovine lung

The 105,000 xg supernatant was dialyzed against 0.154M KCl and 0.05M Tris-HCl buffer pH 7.4 (five changes, one litre each) for 18 hours, at 4^{o}C. The aim of this dialysis was to extract any glutathione present in the cytosol fraction.

The boiled cytosol fraction was prepared by heating a known volume (boiling water bath) of the cytosol fraction for 15 minutes. The protein which precipitates on heating was rehomogenized and the volume corrected for evaporation by adding distilled water.

Homogenization media

0.154M KCl and 0.05M Tris-HCl buffer pH 7.4 was of the following composition:

Tris (hydroxymethyl) methylamine	6.057 g
0.1M HCl	400 ml
KCl	11.634 g

Distilled water was added to make 1 litre.

This medium is recommended by Matsubara, Prough, Burke and Estabrook (1974) for preparation of microsomal fractions.

Phosphate buffer pH 7.4 was of the following composition:

M/5 KH_2PO_4	50 ml
M/5 NaOH	39.5 ml

Distilled water was added to make 200 ml.

Microsomal preparations

Unless otherwise indicated, microsomal preparations used throughout this study were set up as follows:

(1) Pellets of microsomal fractions were rehomogenized in phosphate buffer pH 7.4. Protein concentration was determined, as described previously, and more phosphate buffer was added to the microsomal suspension to adjust protein to the required concentration.

(2) Aliquots (usually 0.5 ml) of microsomal suspension were dispensed in 15 ml capacity, ice cold, test tubes and placed on ice.

(3) Reduced nicotinamide adenine dinucleotide phosphate or a generating system for this compound was added, dissolved in phosphate buffer pH 7.4 (usually in a volume of 0.2-0.5 ml).

(4) Radiolabelled 3-methylindole (specific activity 131 µCi/mmol) was added. A volume of 0.025 ml of 1% "Cremophor-EL" in phosphate

buffer pH 7.4 was used to deliver 3-methylindole.

(5) Reaction was initiated by placing the test tubes in a shaking water bath at 37°C and after incubation the reaction was terminated by placing test tubes on ice and adding 10 volumes of ice cold n-hexane to commence the procedure of organic solvent extraction for determination of covalently bound metabolites.

6.2.4 Determination of microsomal protein

Protein measurement was done using the Folin phenol reagent, as described by Lowry, Rosenburgh, Farr and Randall (1951).

Reagents

Reagent A: 2% Na_2CO_3 in 0.1N NaOH.

Reagent B: 0.5% $CuSO_4$ .5 H_2O in 1% sodium potassium tartrate.

Reagent C: 50 ml of reagent A + 1 ml of reagent B. To be used within 24 hours.

Reagent D: Dilute Folin ciocalteau phenol reagent. Commercially available Folin ciocalteu reagent was diluted with distilled water (1:1:, v/v) to give a concentration of 1N HCl.

Protein standards

A series of standard solutions of horse serum albumin in distilled water were used (0.05-1 mg/ml).

Procedure

Solubilized microsomal proteins were appropriately diluted (usually 1:10 or 1:20) with distilled water. To a sample of protein (0.1 ml) in a 5 ml test tube, 1 ml of reagent C was added, mixed well and allowed to stand for 10 minutes at room temperature. 0.1 ml of

reagent D was added while the test tube continued to be shaken (vortex mixer), mixing continued for further 15 seconds. After 30 minutes the absorbance of the sample was read in a spectrophotometer at 500 mμ wavelength.

Standard protein solutions were similarly treated and a standard curve was established (protein concentration versus absorbance). The concentration of microsomal protein in samples was found from the standard curve.

6.2.5 - Determination of radioactivity covalently bound to microsomal proteins

Microsomal proteins were solubilized in 1N NaOH (boiling water bath) for three minutes. A volume of up to 0.4 ml of the solubilized microsomal protein was pipetted to a liquid scintillation counting vial containing 10 ml of liquid scintillator and 0.5 ml 1N HCl. After tightly sealing the top of the vial, the contents were shaken vigorously.

Radioactivity was measured by liquid scintillation counting using a Packard Tri-carb liquid scintillation spectrometer, model 3255. The addition of HCl to the liquid scintillation cocktail was necessary to neutralize the sodium hydroxide used in solubilizing microsomal protein. Alkaline protein mixtures have a high background counting rate as had been observed during preliminary work. Determination of the counting efficiency was done using an internal standardisation method.

Background radioactivity was determined from vials containing microsomal proteins.

All values were corrected for background radioactivity and quenching.

6.2.6 - Determination of covalent binding of 3-methylindole metabolite(s) to tissue and microsomal proteins

Introduction

The experimental approach adopted in this present work involved administration of radiolabelled forms of 3-methylindole to calves or incubating these radiolabelled forms of 3-methylindole with microsomal preparations. Tissue samples from treated calves or the microsomal suspension were washed repeatedly with organic solvents. This process of exhaustive extraction was designed to remove any free radiolabelled 3-methylindole or 3-methylindole metabolites. Any radioactivity remaining after this organic solvent extraction is presumed to be covalently bound to microsomal or tissue proteins. Finally both the amount of protein and radioactivity associated with the protein were determined. Covalently bound 3-methylindole was calculated and expressed as nmol 3-methylindole/mg microsomal protein. As already discussed, irreversible binding of chemically reactive metabolites to protein does not imply that protein is necessarily the "target substance" but it is the fraction which provides the greatest recovery of covalently bound radioactivity. Therefore measurement of covalent binding to proteins serves as a way of measuring, experimentally, the formation of a metabolite which is sufficiently reactive to alkylate tissue macromolecules. There is no method available in the literature for determination of covalent binding of 3-methylindole to tissue proteins.

Evaluation of methods for determination of covalent binding of radiolabelled 3-methylindole to tissue proteins

Two methods were evaluated for possible use with 3-methylindole. The first method was described by Reid et al (1973) and was used originally for determination of covalent binding of halobenzenes to lung proteins. The method was evaluated as follows:

1 ml boiled microsomal suspension (containing 8 mg protein in phosphate buffer pH 7.4 was mixed with 0.2 ml 1% "Cremophor-EL" containing 0.5 µCi (G-^{3}H) 3-methylindole (specific activity 131 µCi/mmol) in a 15 ml capacity test tube and incubated in a shaking water bath at 37^{o}C for 60 minutes. Test tubes containing only boiled microsomal suspension were included for measurement of background radioactivity. Contents of the test tube were extracted with three volumes of a mixture of n-heptane and isoamyl alcohol (50:1, v/v). Extraction was carried out by shaking for 10 minutes followed by centrifugation at 2000 rpm for three minutes. The n-heptane-isoamyl alcohol extraction was repeated three times. An equal volume of 20% trichloroacetic acid was added after the final heptane-isoamyl alcohol step. The mixture was shaken well and left for 15 minutes. After centrifugation at 2000 rpm for 20 minutes, the trichloroacetic acid was discarded. The protein precipitate was resuspended in 12 ml methanol and shaken for 10 minutes and centrifuged at 2000 rpm for 10 minutes. The methanol extraction was repeated six times.

The protein precipitate was solubilized in 0.6 ml 1N NaOH. 0.1 ml was taken for protein determination and 0.4 ml was taken for radioactivity measurement. Results of duplicate experiments showed

that protein concentration was about 6 mg/ml. The radioactivity associated with protein was about 200 counts per minute, whereas background radioactivity was about 8 counts per minute. Thus this method did not extract radiolabelled 3-methylindole completely from boiled microsomal suspensions. The experiment was repeated on five occasions and gave similar results; therefore it was not considered suitable for use with 3-methylindole.

The method described by Jollow, Mitchell, Potter, Davis, Gillette and Brodie (1973) for determination of covalent binding of radiolabelled acetaminophen to liver proteins was evaluated for use with 3-methylindole.

Duplicate test tubes containing microsomal suspension and tritiated 3-methylindole were prepared as described above. An equal volume of 0.9M trichloroacetic acid was added to the microsomal suspension and shaken well. The protein was precipitated by centrifugation at 2000 rpm for 20 minutes. The supernatant was discarded and the protein precipitate was resuspended in 10 ml of 0.6M trichloroacetic acid, shaken well and then centrifuged at 2000 rpm for five minutes. The supernatant was discarded and the protein washed twice with further amounts of 0.6M trichloroacetic acid.

The protein was resuspended in 12 ml of 60% methanol, mixed for three minutes and the supernatant discarded. This procedure was repeated six times. After extraction the protein precipitate was solubilized in 0.6 ml 1N NaOH. Aliquots were taken for protein determination (0.1 ml) and liquid scintillation counting (0.4 ml). Results of duplicate experiments showed that protein concentration was

about 8 mg/ml, radioactivity associated with protein was about 250 counts per minute, whereas background radioactivity was about 8 counts per minute. The experiment was repeated on three occasions and gave similar results.

The possibility that the radioactivity remaining after organic solvent extraction, using the two methods described, was due to non-specific tritium exchange between tritiated 3-methylindole and microsomal proteins was investigated by using (methyl ^{14}C) 3-methylindole. Both methods of extraction again failed to extract all radioactivity from the ^{14}C labelled 3-methylindole, suggesting that radioactivity from tritiated or ^{14}C labelled 3-methylindole is not due to non-specific tritium exchange or carbon incorporation.

The possibility that failure of the above described methods to extract radiolabelled 3-methylindole was due to a non-enzymatic covalent reaction between a labelled contaminant of labelled 3-methylindole and microsomal proteins was also investigated by incubating 2 mmol of ^{14}C labelled 3-methylindole with 20 mg of boiled microsomal proteins (in 1 ml phosphate buffer pH 7.4) for three hours at 37^{o}C. ^{14}C labelled 3-methylindole was extracted with 5 ml hexane. The hexane was pipetted into 15 ml capacity test tubes and evaporated (40^{o}C, under nitrogen). Boiled microsomal suspension (1 ml) was added (containing 8 mg protein in phosphate buffer pH 7.4) and shaken well. The contents of the test tube were subjected to extraction by the method of Reid et al (1973) and by the method of Jollow, Mitchell, Potter, Davis, Gillette and Brodie (1973). This did not achieve complete extraction of radioactivity by either method, excluding the possibility of a radiolabelled contaminant to 3-methylindole.

Procedure used for the determination of covalent binding of radiolabelled 3-methylindole to tissue proteins

The following method was developed after an investigation of the solubility of 3-methylindole in various solvents and after an evaluation of their usefulness in extracting 3-methylindole added to microsomal protein preparations.

Test tubes containing microsomal preparations were placed on ice. (to inhibit enzymetic activity), and the microsomal suspension was extracted with 3 x 10 volumes of cold ($0^{o}C$) n-hexane, then with 2 x 10 volumes ethyl acetate. Extraction was carried out by vigorous vortex shaking for two minutes. The organic phases were aspirated (pasteur pipette) and discarded. While the test tube continued to be shaken on the vortex shaker, an equal volume of 20% trichloroacetic acid was added, followed immediately by the addition of 10 ml absolute ethanol, with continuous vortex shaking for three minutes. After centrifugation for 15 minutes at 3000 rpm, the trichloroacetic acid ethanol phase was discarded.

The protein precipitate was resuspended in 10 ml absolute ethanol, shaken for five minutes and centrifuged at 2000 rpm for five minutes. After centrifugation the ethanol layer was discarded. The ethanol extraction was repeated four times. The protein precipitate was extracted with 3 x 10 ml methanol as described for ethanol. The protein precipitate was solubilized in 1N NaOH and aliquots were taken for liquid scintillation counting and protein determination.

Blanks

1 ml boiled microsomal suspension (containing 8 mg protein

in phosphate buffer pH 7.4) was mixed with 0.2 ml 1% "Cremophor-EL" containing 0.5 µCi (G-^{3}H) 3-methylindole in a 15 ml capacity test tube and incubated in a shaking water bath at 37^{o}C for 60 minutes. Test tubes containing only boiled microsomal suspension were included for determination of background radioactivity. Contents of test tubes were subjected to organic solvent extraction as described above. The protein precipitate was solubilized in 0.6 ml 1N NaOH. Aliquots were taken for liquid scintillation counting (0.4 ml) and protein determination (0.1 ml).

Results of duplicate experiments were:
Protein concentrations were 5.8 and 6.0 mg/ml, blank radioactivities were 12 and 14 counts per minute and background radioactivities were 8 and 9 counts per minute.

Blanks containing boiled microsomes and radiolabelled 3-methylindole were included with each experiment. Blank values of radioactivity never exceeded double the background counts per minute of the liquid scintillation spectrometer.

6.2.7 - Studies on the covalent binding of 3-methylindole to bovine tissues in vivo

Animals

Three Ayrshire breed calves (50-60 kg body weight) were anaesthetized with pentobarbitone (20 mg/kg body weight) with additional 2 mg/kg doses given intravenously as required throughout the course of the experiments to maintain anaesthesia.

3-Methylindole solution

Tritiated 3-methylindole of specific activity of 20 µCi/mmol

was dissolved (30 mg/ml) in 10% "Cremophor-EL" in distilled water.

Infusion of 3-methylindole

Infusions were made into the femoral vein via an indwelling cannula. Each dose was infused over one minute with the subsequent doses given after 30 minutes.

Tissue samples

Calves surviving the experimental procedure were killed (exsanguinated by a jugular section) two and a half hours after the last 3-methylindole infusion. Tissue samples were taken from the lung, heart, liver, kidney, cerebral hemispheres, skeletal muscles (chest and thigh) and perirenal fat.

Total and covalently bound radioactivities were determined.

Determination of total radioactivity

Tissue was digested by placing 1 g of tissue in a Teflon lined stainless steel pressure vessel (R. A. Scientific, 34 Bolton Gardens, London, England). 1 ml concentrated hydrochloric acid and 2 ml hydrogen peroxide (30% v/v) were added to the homogenate and the vessel tightly sealed. Digestion was carried out by placing the vessel in a boiling water bath for two hours. The vessel was cooled and the final volume of the solubilized tissue was measured. 1 ml of the solubilized tissue was added to 12 ml of liquid scintillator Unisolve 1 in a liquid scintillation vial. The vial was sealed and shaken well. Vials were left overnight at $4^{o}C$ in the dark. Radioactivity was measured using liquid scintillation counting.

All values were corrected for background and quenching, using internal standardization method.

Determination of covalently bound radioactivity

1 g of tissue was homogenized in three volumes of water (M.S.E. tissue homogenizer, M.S.E. Ltd., Manor Royal, Crawley, Sussex, England). Tissue homogenates were subjected to extensive organic extraction as described for microsomes. Extracted tissue homogenates were dried to a constant weight at 60°C. 100 mg dry tissue homogenate were digested in 1 ml concentrated hydrochloric acid and 2 ml hydrogen peroxide. The remainder of the procedure was as described for determination of total radioactivity.

Results

One of the calves (Calf 1) survived five doses of tritiated 3-methylindole (1,1,2,4 and 12 mg/kg) and was killed two and a half hours after the last injection. One of the other two calves (Calf 2) died two minutes after the last of three doses of 3-methylindole (1, 1 and 2 mg/kg) and the remaining calf (Calf 3) died two minutes after a dose of 1 mg/kg of 3-methylindole.

Table 6.1 shows the concentration of total and covalently bound radioactivity in different tissues of the three calves. The greatest concentration of covalently bound radioactivity occurred in the lung in all three calves.

Blank values were obtained by adding 0.2 µCi of (G-^{3}H) 3-methylindole to 1 g of normal lung homogenate at 1°C and subjecting to the same treatment described above for determination of covalently bound radioactivity in tissues. Blank values were less than 12 counts per minute. This value was subtracted before calculating the concentration of covalently bound radioactivity.

TABLE 6.1 CONCENTRATION OF TOTAL (nmol 3-METHYLINDOLE/g TISSUE WET WEIGHT) AND COVALENTLY BOUND (nmol 3-METHYLINDOLE/g DRIED EXTRACTED TISSUE HOMOGENATE) RADIOACTIVITY IN TISSUES OF THREE CALVES AFTER ADMINISTRATION OF TRITIATED 3-METHYL-INDOLE. EACH VALUE REPRESENTS THE MEAN OF TWO ESTIMATIONS. S.E. WAS WITHIN 15% OF THE MEAN

	Calf 1		Calf 2		Calf 3	
	Total	Bound	Total	Bound	Total	Bound
Skeletal muscle	87	4	12	0	3	0
Heart muscle	64	11	129	9	160	27
Lung	264	913	257	740	258	603
Kidney	312	365	358	320	169	49
Liver	240	675	368	370	52	20
Fat	189	-	280	-	70	-

6.2.8 - *In vitro* studies on the enzyme system catalyzing metabolic activation of 3-methylindole

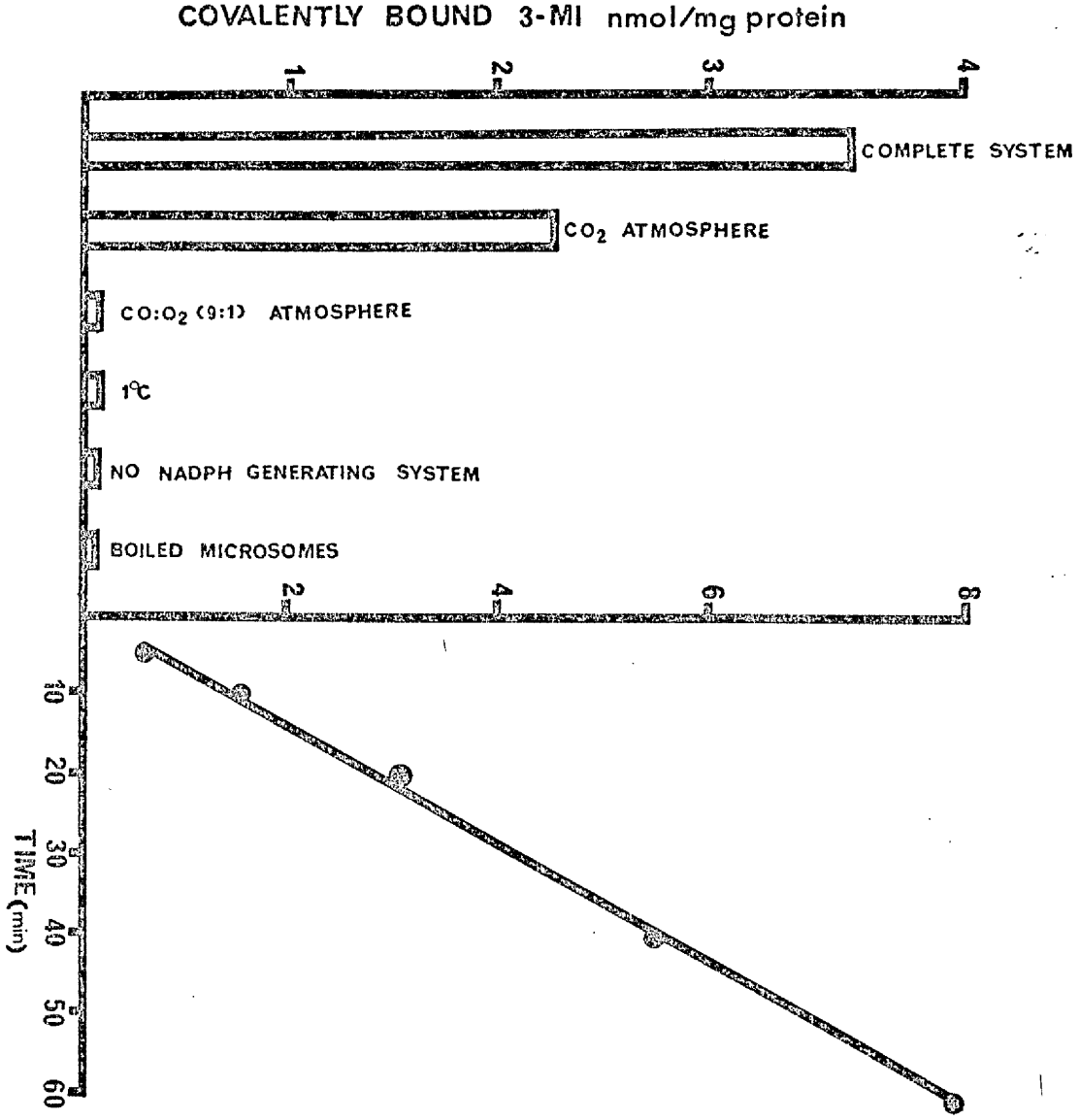
Introduction

In vivo studies reported in this chapter indicate that the metabolism of 3-methylindole in cattle involves the formation of a reactive metabolite(s) which becomes covalently bound to tissue proteins. As discussed earlier in this chapter, microsomal enzymes can catalyze the bioactivation of a wide variety of foreign compounds including pulmonary toxic furano compounds and halobenzenes to chemically reactive metabolites. This present work was undertaken to investigate the enzyme system catalyzing covalent binding of 3-methylindole to tissue proteins, using microsomal preparations from the bovine lung.

Determination of the metabolic requirements for covalent binding of 3-methylindole to microsomal proteins of the bovine lung

Incubations were run in duplicate using a total volume per incubation of 1 ml of phosphate buffer pH 7.4 containing 5 mg lung microsomal protein, 0.5 µmol (methyl-^{14}C) 3-methylindole (131 µCi/mmol) and a reduced nicotinamide adenine dinucleotide phosphate-generating system consisting of 8.6 mg nicotinamide adenine dinucleotide phosphate, 63 mg glucose-6-phosphate and 2 units of glucose-6-phosphate dehydrogenase. Boiled microsomes were used in a duplicate incubation and other duplicate incubations did not contain the reduced nicotinamide adenine dinucleotide phosphate-generating system. Incubations were done in a shaking water bath at 37^{o}C for 30 minutes under an air atmosphere unless otherwise indicated. Some

Fig. 6.2 The extent of covalent binding of 3-methylindole (3MI) metabolites to bovine lung microsomes in the presence of different incubation conditions and also using the complete system with time


COVALENTLY BOUND 3-MI nmol/mg protein
1
2
3
4
COMPLETE SYSTEM
CO_2 ATMOSPHERE
$CO:O_2$ (9:1) ATMOSPHERE
1°C
NO NADPH GENERATING SYSTEM
BOILED MICROSOMES
2
4
6
8
10
20
30
40
50
60
TIME (min)

incubations were done under a carbon dioxide atmosphere or a carbon monoxide-oxygen (9:1) atmosphere or were done at 1°C.

At the end of the incubation test tubes were placed on ice and assayed for covalently bound 3-methylindole metabolites as described previously.

Results

Covalent binding was optimal when fresh microsomes were incubated with the reduced nicotinamide adenine dinucleotide phosphate-generating system, under air atmosphere, at 37°C (complete system). Covalent binding was strongly inhibited in the absence of reduced nicotinamide adenine dinucleotide phosphate, in boiled microsomes, and when incubation was made at 1°C, under carbon dioxide atmosphere, or under a carbon monoxide enriched atmosphere. Fig. 6.2 shows the extent of covalent binding of 3-methylindole metabolite(s) to microsomal protein under different incubation conditions.

Effect of the concentration of reduced nicotinamide adenine dinucleotide phosphate on the extent of covalent binding of 3-methylindole to microsomal proteins

Duplicate incubations each contained 3 mg lung microsomal proteins, 0.25 µmol (G-^{3}H) 3-methylindole and the appropriate amount of reduced nicotinamide adenine dinucleotide phosphate in 1 ml phosphate buffer (pH 7.4). Concentrations of reduced nicotinamide adenine dinucleotide phosphate in different incubations were 0, 0.5, 1, 2, 4, 6 and 8 µmol/ml. Incubation was done in a shaking bath at 37°C for 20 minutes. Assay for covalently bound metabolites was done as

described previously.

Results

Covalent binding to microsomal proteins was dependent on the concentration of reduced nicotinamide adenine dinucleotide phosphate, and reached a maximal magnitude at a concentration of 8 mmol.

The extent of covalent binding of 3-methylindole metabolite(s) to microsomal proteins at different concentrations of reduced nicotinamide adenine dinucleotide phosphate is shown in Fig. 6.3.

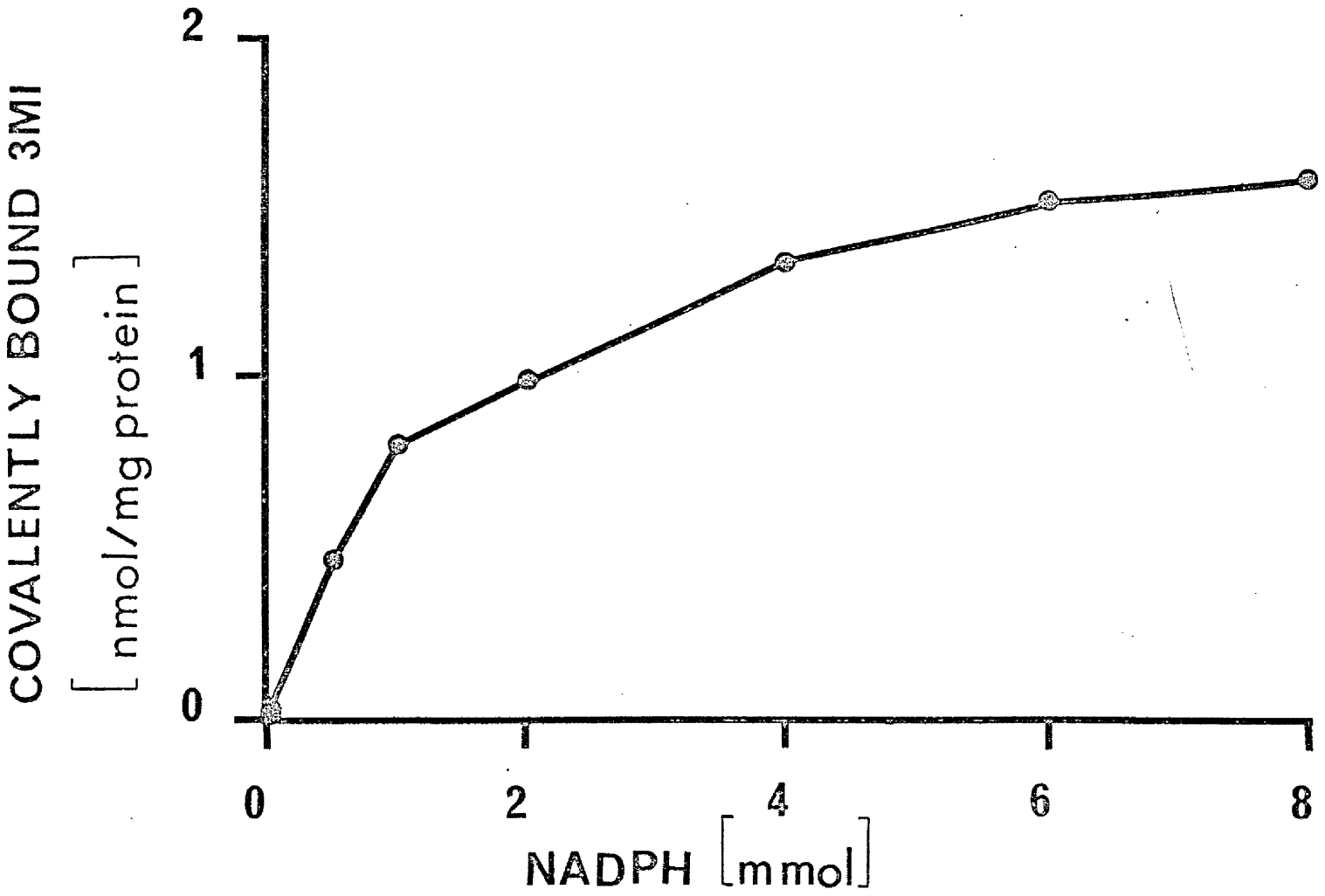
Effect of time on the extent of covalent binding of 3-methylindole metabolite(s) to microsomal proteins

A total volume of incubation mixture containing 44 mg lung microsomal protein, 40 mg nicotinamide adenine dinucleotide phosphate, 315 mg glucose-6-phosphate, 7 units glucose-6-phosphate dehydrogenase and 2 µmol (G-^{3}H) 3-methylindole (131 µCi/mmol), in phosphate buffer pH 7.4 in 25 ml conical flask, were incubated in a shaking water bath at 37^{o}C under air atmosphere. Duplicate 0.5 ml samples were taken after 5, 10, 20, 40 and 60 minutes. The samples were pipetted into ice cold 10 ml capacity test tubes. Enzymatic activity was stopped by adding 10 volumes of ice cold hexane and assay for covalently bound 3-methylindole metabolites was carried out.

Results

The results are shown in Fig. 6.2. The covalent binding of 3-methylindole to microsomal protein increased linearly during a one hour incubation.

Fig. 6.3 The extent of covalent binding of 3-methylindole metabolite to microsomal proteins in the presence of different concentrations of reduced nicotinamide adenine dinucleotide phosphate (NADPH)


COVALENTLY BOUND 3MI
[nmol/mg protein]
2
1
0
0
2
4
6
8
NADPH [mmol]

Effect of L-cysteine, cytochrome c and SKF-525A on the extent of covalent binding of 3-methylindole metabolite(s) to microsomal protein

Duplicate incubations each contained 4 mg lung microsomal protein, 8.5 mg nicotinamide adenine dinucleotide phosphate, 63 mg glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 0.5 µmol (G-^{3}H) 3-methylindole (131 µCi/mmol) and the appropriate concentration of cytochrome c, or SKF-525A or L-cysteine if required in a total volume of 2 ml phosphate buffer pH 7.4. Incubations containing no inhibitors and blank incubations containing boiled microsomes only were included. Incubation was made at 37^{o}C for 30 minutes. Assay for covalently bound metabolites was carried out as described previously.

Results

As shown in Table 6.2, addition of L-cysteine or cytochrome c or SKF-525A caused a concentration dependent inhibition of covalent binding.

Studies on the liver microsomal enzyme system catalyzing the covalent binding of 3-methylindole

In vivo studies indicate that 3-methylindole becomes covalently bound to proteins of the hepatic tissues in vivo. It was of interest to elucidate the liver enzyme system catalyzing this covalent binding and to know whether the hepatic and pulmonary enzyme systems are qualitatively similar. The experimental design was as described previously for the lung. In addition some incubations contained SKF-525A (4 µmol/ml) and cytochrome $_{c}$ (50 nmol/ml).

TABLE 6.2 THE PERCENTAGE INHIBITION OF COVALENT BINDING OF TRITIATED 3-METHYLINDOLE METABOLITES TO BOVINE LUNG MICROSOMES WITH INCREASING CONCENTRATIONS OF CYTOCHROME c, SKF-525A and L-CYSTEINE

SKF-525A		Cytochrome c		L-cysteine	
Concentration (µmol/ml)	% Inhibition	Concentration (nmol/ml)	% Inhibition	Concentration (µmol/ml)	% Inhibition
0.1	14	5	4	2	0
2	64	25	30	4	10
4	74	50	41	8	25
8	89	250	93	16	40

Results

Covalent binding was optimal when fresh microsomes were incubated with the reduced nicotinamide adenine dinucleotide phosphate-generating system, under air atmosphere, at 37°C. Covalent binding was strongly inhibited in the absence of the reduced nicotinamide adenine dinucleotide phosphate-generating system, in boiled microsomes, and when incubation was made at 1°C, or under carbon dioxide atmosphere or carbon monoxide enriched atmosphere. Addition of SKF-525A or cytochrome c inhibited covalent binding. Fig. 6.4 shows the extent of covalent binding of 3-methylindole metabolite(s) to microsomal proteins under different incubation conditions.

6.2.9 - Comparison of the covalent binding of indole, 3-methylindole and 3-methyloxindole

Duplicate incubations each containing 4 mg lung microsomal proteins, 8.6 mg nicotinamide adenine dinucleotide phosphate, 63 mg glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase and 0.5 µmol (G-^{3}H) indole or (G-^{3}H) 3-methylindole or (G-^{3}H) 3-methyloxindole in 1 ml phosphate buffer pH 7.4 were incubated in a shaking water bath at 37°C for 60 minutes. Duplicate blank incubations containing boiled microsomes were also included. Assay for covalently bound metabolites was carried out as described previously.

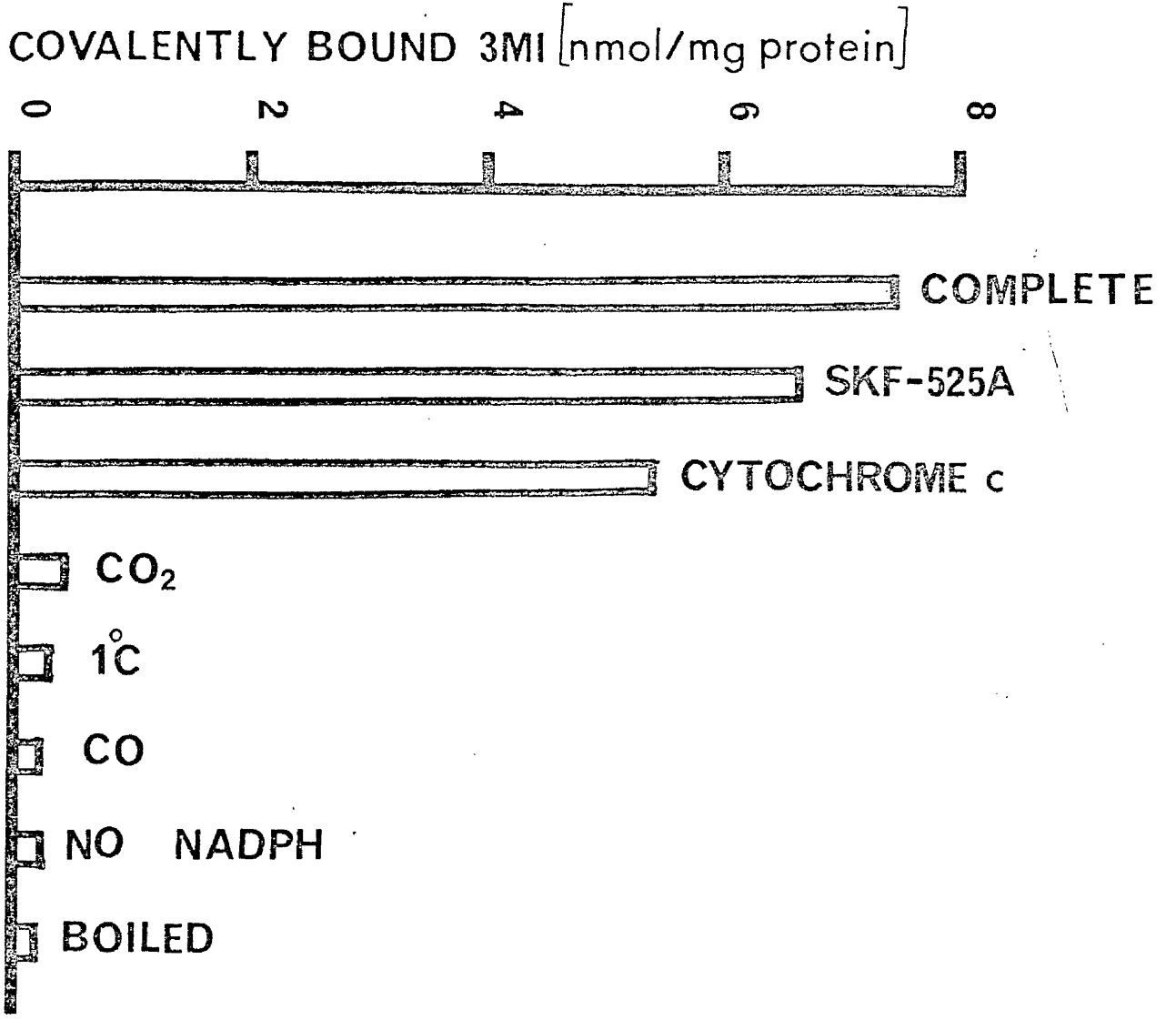
Results

As shown in Table 6.3 3-methylindole became covalently bound to microsomal proteins while indole and 3-methyloxindole did not become covalently bound to microsomal proteins to any great extent.

TABLE 6.3 COVALENT BINDING OF (G_3-^{3}H) 3-METHYLINDOLE, (G-^{3}H) METHYLOXINDOLE AND (G-^{3}H) INDOLE TO BOVINE LUNG MICROSOMES (DUPLICATE EXPERIMENTS)

Substrate	Covalently bound radioactivity (mean ± S.E.) (nmol/mg protein)	
	Fresh microsomes	Boiled microsomes
3-methylindole	14.48 ± 0.19	0.12 ± 0.05
3-methyloxindole	0.17 ± 0.04	0.19 ± 0.03
Indole	0.15 ± 0.05	0.11 ± 0.08

Fig. 6.4 The extent of covalent binding of 3-methylindole metabolites to liver microsomes under different incubation conditions


COVALENTLY BOUND 3MI [nmol/mg protein]
0
2
4
6
8
COMPLETE
SKF-525A
CYTOCHROME c
CO_2
1°C
CO
NO NADPH
BOILED

6.2.10 - *In vitro* studies on the nature of the reactive metabolite of 3-methylindole

Preliminary work reported earlier in this chapter showed that L-cysteine inhibited the covalent binding of 3-methylindole metabolite to microsomal proteins (*in vitro*) suggesting that the reactive metabolite of 3-methylindole is electrophilic. The present work was undertaken to identify the nature of the reactive intermediate using three nucleophilic agents, glutathione, L-cysteine and N-acetyl-L-cysteine. The possible role of glutathione ^{S}-transferases in the detoxification of the 3-methylindole reactive metabolite was also investigated using the cytosol fraction obtained from the bovine lung as a source of this enzyme.

Effect of various concentrations of glutathione on the covalent binding of 3-methylindole to microsomal proteins

1 ml phosphate buffer pH 7.4 containing 5 mg washed lung microsomal proteins, 8.5 mg reduced nicotinamide adenine dinucleotide phosphate, 63 mg glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase, 0.25 µmol (G-^{3}H) 3-methylindole (131 µCi/mmol) and the appropriate amount of glutathione were incubated at 37^{o}C for 30 minutes. Incubations were run in duplicate. Assay for covalently bound metabolites was conducted as described before.

Results

As shown in Fig. 6.5, glutathione caused concentration dependent inhibition of covalent binding of radioactivity to microsomal protein. Complete inhibition (99% inhibition) of covalent binding was achieved at a concentration of 64 mmol glutathione.

Effect of the cytosol fraction and nucelophilic agents on the covalent binding of 3-methylindole to microsomal protein.

Duplicate incubations were used; each contained:

0.25 ml washed lung microsomal suspension in phosphate buffer, pH 7.4 (containing 5 mg protein)

+ 0.25 ml unboiled or boiled lung cytosol fraction (containing 1.5 mg protein)

+ 0.28 ml phosphate buffer, pH 7.4, containing the appropriate amount of glutathione or cysteine or N-acetyl-L-cysteine. In some incubations the phosphate buffer alone was added.

+ 0.2 ml phosphate buffer, pH 7.4, containing 8.5 mg nicotinamide adenine dinucleotide phosphate + 63 mg glucose-6-phosphate and 2 units glucose-6-phosphate dehydrogenase.

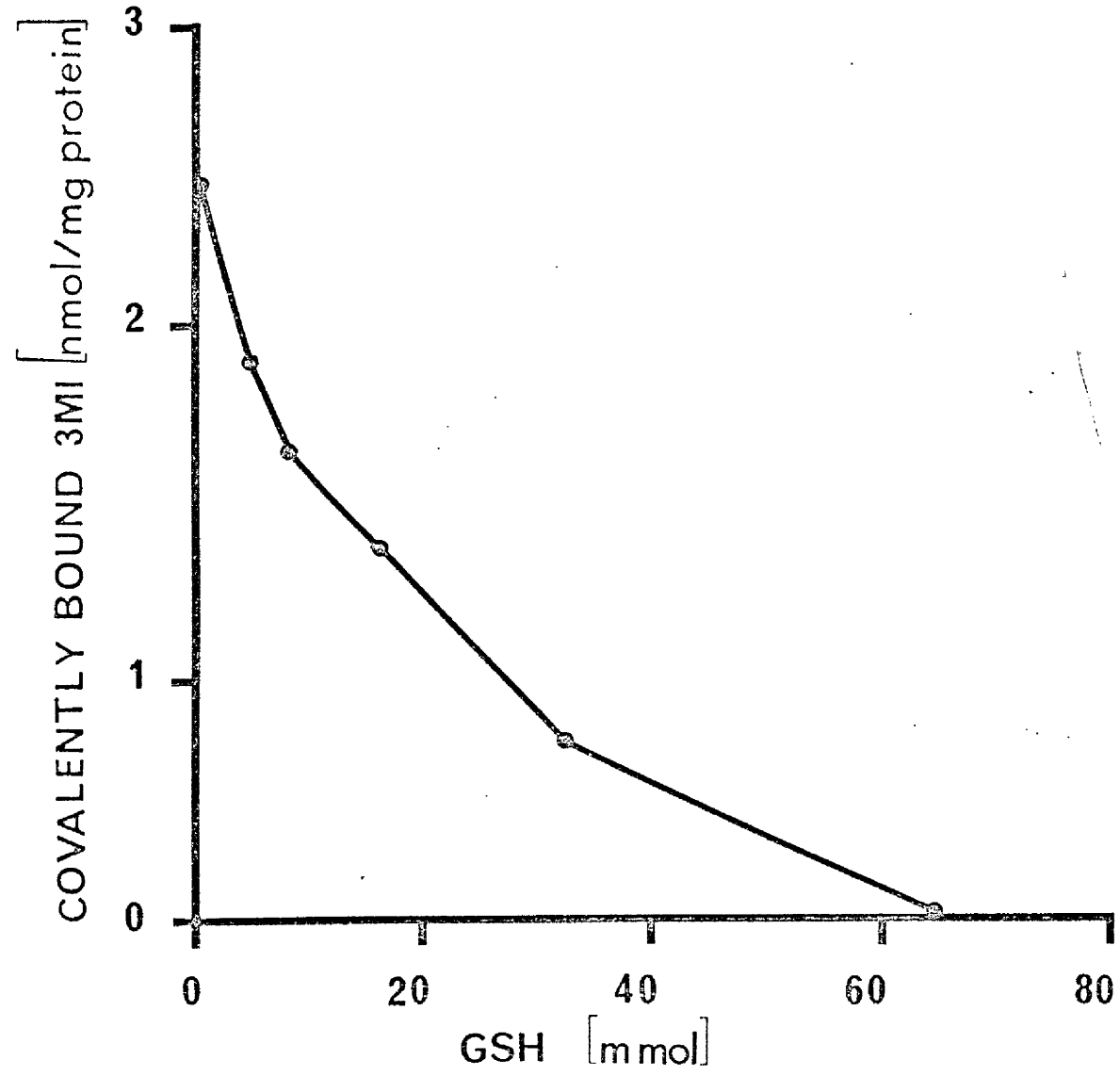
+ 0.02 ml of 1% "Cremophor-EL" in phosphate buffer, pH 7.4, containing 0.25 µmol (G-^{3}H) 3-methylindole (131 µCi/mmol).

Incubations were run for 20 minutes at 37^{0}C in a shaking water bath. Assay for covalently bound metabolites was carried out as described before.

Results

As shown in Fig. 6.6 addition of unboiled lung cytosol alone inhibited covalent binding to microsomal proteins, compared with the boiled cytosol fraction. Addition of increasing concentrations of glutathione, L-cysteine or N-acetyl-L-cysteine together with boiled lung cytosol caused concentration dependent inhibition of covalent binding to microsomal proteins. Addition of unboiled lung cytosol together with glutathione or L-cysteine

Fig. 6.5 The extent of covalent binding of 3-methylindole (3MI) metabolites to lung microsomal proteins in the presence of different concentrations of glutathione (GSH)


COVALENTLY BOUND 3MI [nmol/mg protein]
3
2
1
0
0
20
40
60
80
GSH [m mol]

or N-acetyl-L-cysteine caused a greater extent of inhibition of covalent binding. The differences between boiled and unboiled cytosol fractions were greater with glutathione than with L-cysteine or N-acetyl-L-cysteine.

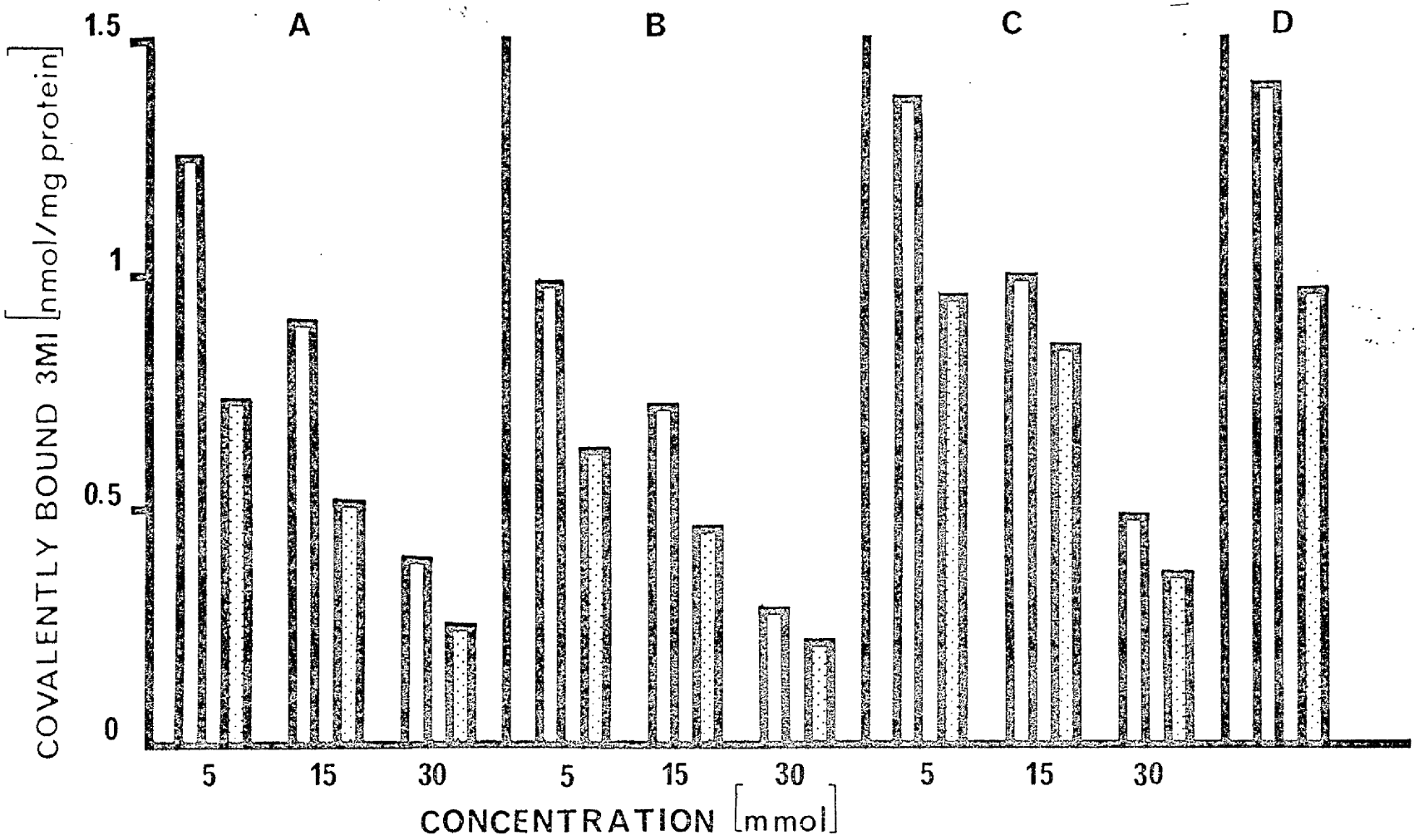
6.2.11 - Studies on organ specificity of 3-methylindole

Introduction

It has been established that 3-methylindole causes specific pulmonary toxic effect in cattle. The gradual accumulation of information indicates that the pulmonary toxic effect of 3-methylindole is based on its metabolism to a reactive electrophilic metabolite. The enzyme system catalyzing the activation of 3-methylindole, viz cytochrome P-450 dependent mixed function oxidase is present in extra pulmonary tissue, such as the liver and kidney. The reason why 3-methylindole is not toxic to other organs is not known. A possible answer for this question is that the rate of reactive metabolite formation from 3-methylindole is greater in lung than in the liver or the kidney. This possibility was investigated by comparing the rate of reactive metabolite formation, from 3-methylindole, by microsomal preparations from the lung, liver and kidney.

In this present work microsomal preparations derived from the lung, liver and kidney of the same group of animals (four animals) were used in each experiment. Separate experiments were performed on microsomal preparations from different groups.

Fig. 6.6 Effect of glutathione, cysteine and N-acetyl-L-cysteine and the lung cytosol fraction on covalent binding to microsomal proteins. Tritiated 3-methylindole (3MI) was incubated with lung microsomes, a reduced nicotinamide adenine dinucleotide phosphate-generating system, unboiled (stippled bars) or boiled (open bars) lung cytosol, and either glutathione (A), L-cysteine (B) or N-acetyl-L-cysteine (C) at three concentrations (5, 15 and 30 mmol). None of these three compounds was added to incubations D.


COVALENTLY BOUND 3MI [nmol/mg protein]
1.5
1
0.5
0
A
B
C
D
5
15
30
5
15
30
5
15
30
CONCENTRATION [mmol]

Time sequence of covalent binding of 3-methylindole to proteins of of microsomal preparations from the lung, liver and kidney

Microsomal fractions from the liver and kidney were obtained as described previously for the lung.

A total volume of 6 ml contained microsomal suspension (48 mg protein) from the lung, liver or kidney, 40 mg nicotinamide adenine dinucleotide phosphate, 315 mg glucose-6-phosphate, 7 units glucose-6-phosphate dehydrogenase and 3 µmol (G-^{3}H) 3-methylindole (131 µCi/mmol), in phosphate buffer, pH 7.4. The mixture was incubated in a 25 ml capacity flask in a shaking water bath at 37^{o}C under air atmosphere. Duplicate 0.5 ml samples were taken after 5, 10, 20, 40 and 60 minutes. The samples were pipetted into ice cold 10 ml capacity test tubes. Assay for covalently bound 3-methylindole metabolites was started immediately after sampling.

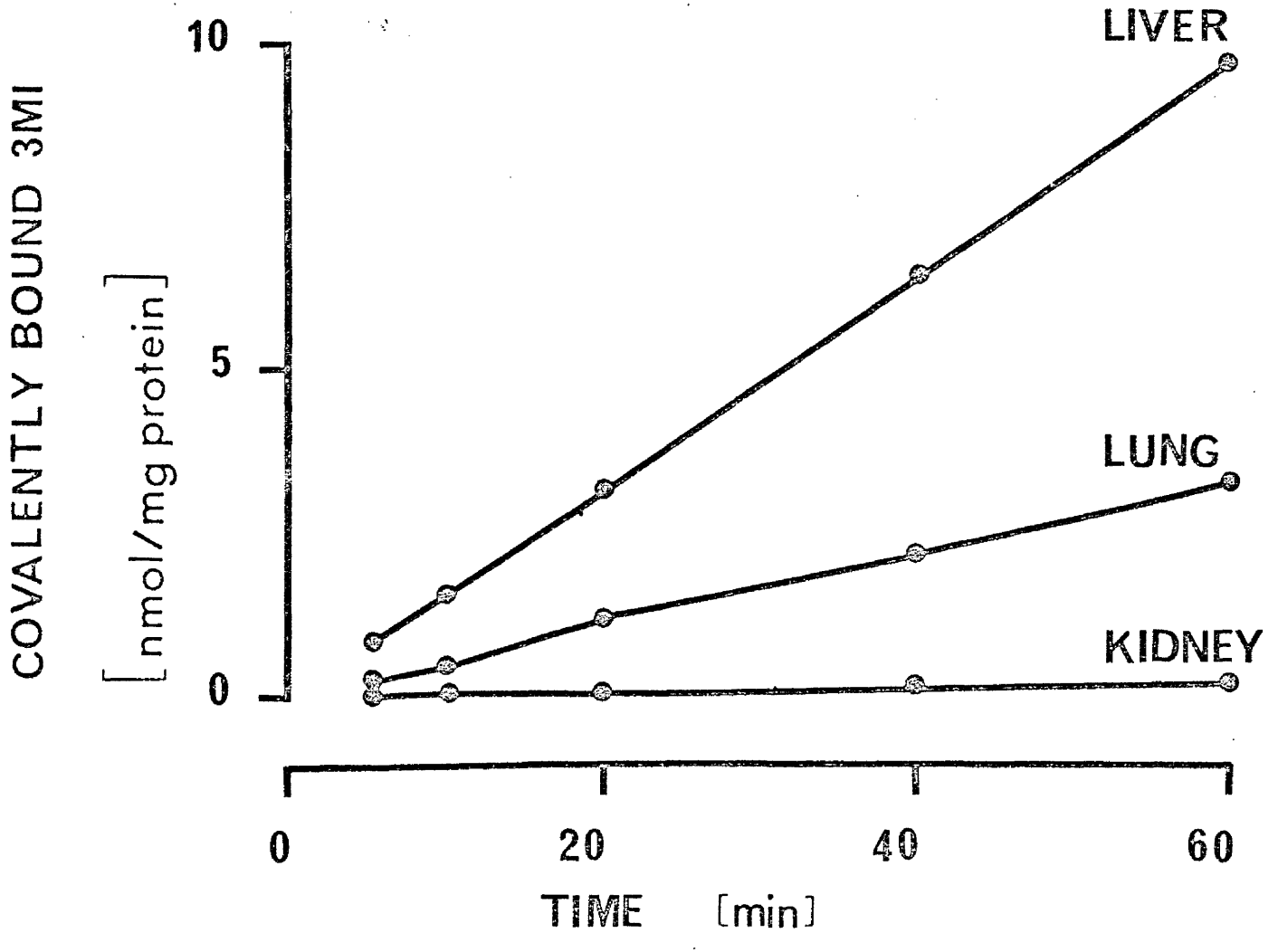
Results

As shown in Fig. 6.7 covalent binding of 3-methylindole to microsomal proteins increased linearly during a one hour incubation. The magnitude of covalent binding to microsomal preparations from the liver was the greatest followed by those from the lung. Microsomal preparations from the kidney showed the least covalent binding and therefore kidney microsomal preparations were not used in the subsequent experimental work.

Effect of concentration of microsomal protein on the rate of covalent binding of 3-methylindole to microsomal proteins

Duplicate incubations of lung and liver microsomal preparations were used. A series of dilutions of lung and liver

Fig. 6.7 Time sequence of covalent binding of 3-methylindole (3MI) to liver, lung and kidney microsomal protein during a one hour incubation


COVALENTLY BOUND 3MI
[nmol/mg protein]
10
5
0
LIVER
LUNG
KIDNEY
0
20
40
60
TIME [min]

microsomal suspensions were prepared in phosphate buffer, pH 7.4 (6, 5, 4, 3, 2 and 1 mg microsomal protein/ml).

0.5 ml aliquots of microsomal suspensions were dispensed into 15 ml capacity test tubes (ice cold). 0.5 ml phosphate buffer, pH 7.4, containing 8.5 mg nicotinamide adenine dinucleotide phosphate, 63 mg glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase and 0.5 µmol (G-^{3}H) 3-methylindole (131 µCi/mmol) were added to each test tube and these were incubated at 37^{o}C for 30 minutes. Assay for covalently bound metabolites was carried out as described previously.

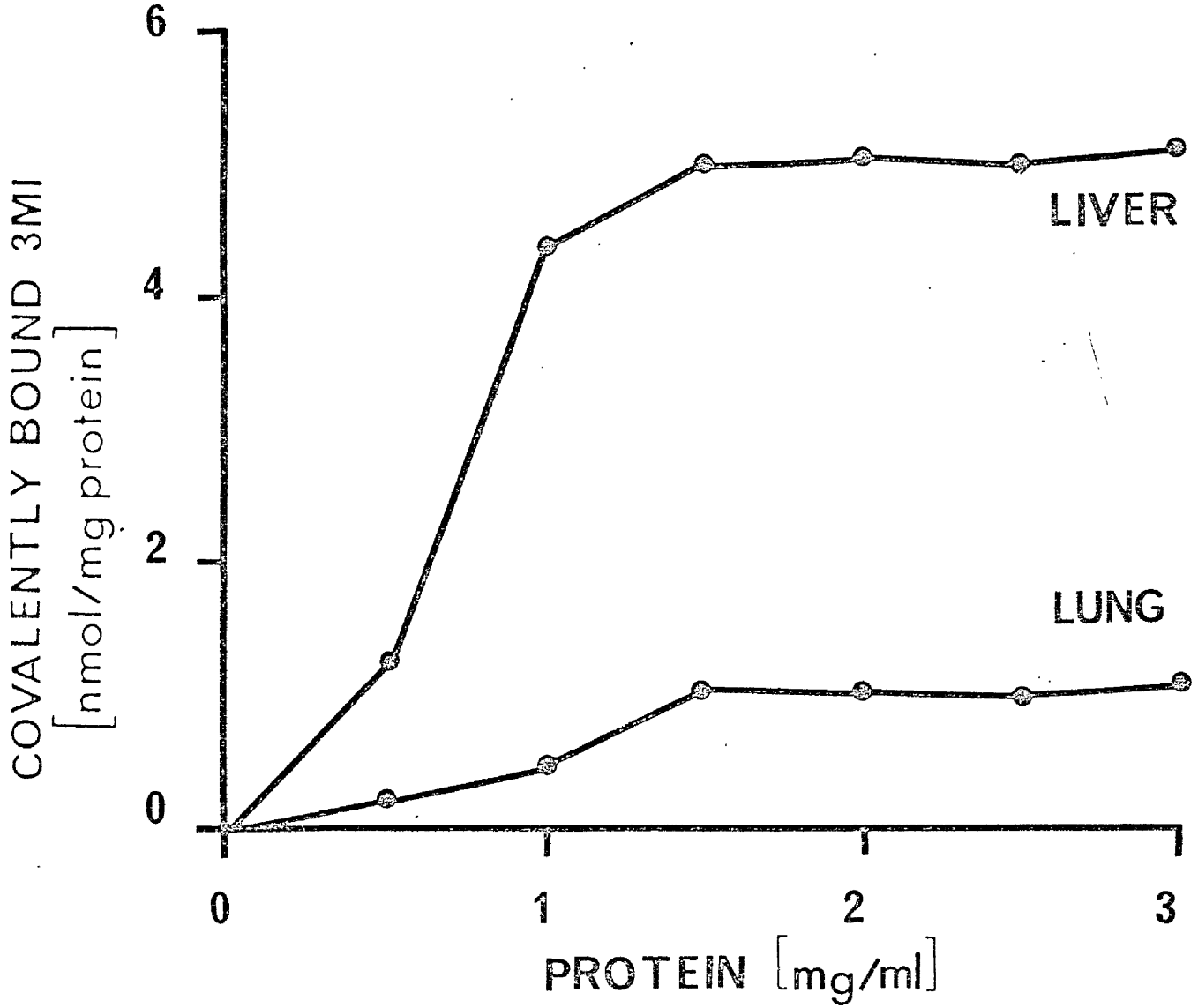
Results

As shown in Fig. 6.8 the magnitude of covalent binding was dependent on the concentration of microsomal protein and reached a maximal at a protein concentration of 1.5 mg/ml. At all protein concentrations the magnitude of covalent binding to liver microsomal proteins was greater than to lung microsomal proteins.

Measurement of kinetic constants for the hepatic and pulmonary microsomal enzyme systems catalyzing covalent binding of 3-methylindole

The dependence of velocity of enzyme catalyzed reactions on substrate concentration is important in enzyme kinetics. This property can be investigated by determining the Michaelis constant (Km) which is equal to the substrate concentration giving half-maximal velocity. The maximal velocity (V_{max}) of an enzyme reaction is the velocity obtained when the substrate concentration is sufficiently high to saturate the enzyme. Both V_{max} and Km can be determined from a series of measurements of velocity at different substrate concen-

Fig. 6.8 The extent of covalent binding of 3-methylindole (3MI) metabolites to liver and lung microsomal protein at different microsomal protein concentrations


6
4
2
0
COVALENTLY BOUND 3MI
[nmol/mg protein]
LIVER
LUNG
0
1
2
3
PROTEIN [mg/ml]

trations. To determine these constants for the lung and liver enzyme systems microsomal preparations were incubated with a reduced nicotinamide adenine dinucleotide phosphate-generating system and different concentrations of (G-^{3}H) 3-methylindole. Assay for covalently bound metabolites was carried out. Velocity (V) was calculated (pmol 3-methylindole/mg protein/minute) at different 3-methylindole concentration(s). The reciprocal of velocity ($\frac{1}{V}$) was plotted against the reciprocal of the substrate (3-methylindole) concentration ($\frac{1}{S}$). This is known as the Lineweaver-Burk plot and is one of the graphical methods for the determination of the enzyme kinetic constants. If $\frac{1}{V}$ is plotted against $\frac{1}{S}$ a straight line is obtained. This straight line cuts the base line at a point giving $-\frac{1}{Km}$ and cuts the vertical axis at a point which gives $\frac{1}{Vmax}$ and has a slope of $\frac{Km}{Vmax}$ (Dixon and Webb, 1964).

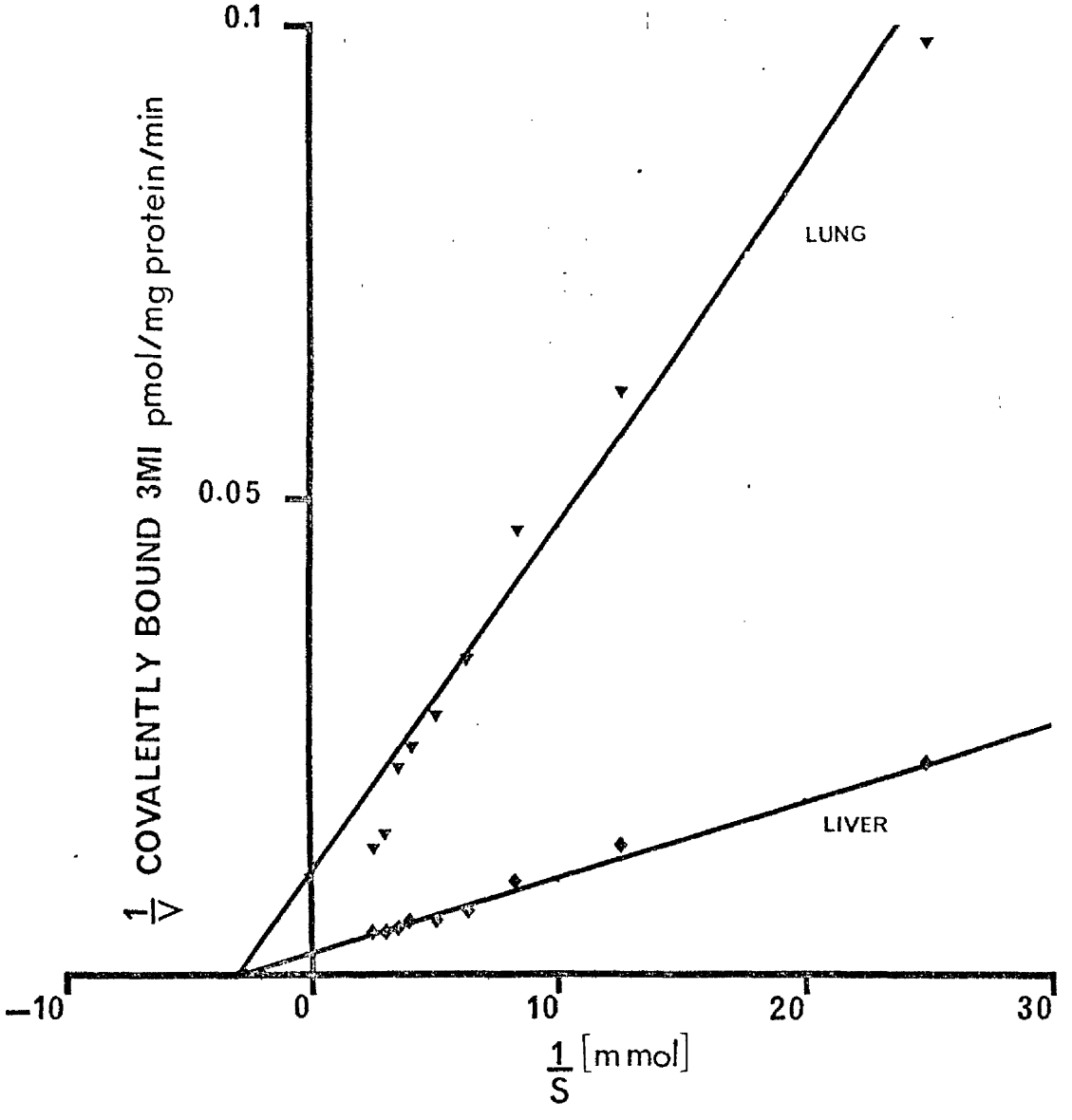
Microsomal preparations were set up as follows:

Triplicate tubes were used. Each incubation tube contained liver or lung microsoral suspension (4 mg protein), 8.6 mg nicotinamide adenine dinucleotide phosphate, 63 mg glucose-6-phosphate and 2 units glucose-6-phosphate dehydrogenase and the appropriate amount of (G-^{3}H) 3-methylindole (131 µCi/mmol), in a total volume of 1 ml phosphate buffer, pH 7.4. Incubation was carried out for 30 minutes in a shaking water bath at 37^{o}C. The reaction was stopped by placing test tubes on the ice and adding 10 ml ice cold n-hexane to start the procedure for assay for covalently bound metabolites.

Results

A plot of the reciprocal of velocity of covalent binding

Fig. 6.9 Lineweaver-Burk plot of the reciprocal of the velocity of covalent binding against the reciprocal of 3-methylindole concentration. Values represent the mean of three experiments; the standard error of the mean was within 5% of the mean. The regression line was fitted by the method of least squares.


0.1
0.05
1/V COVALENTLY BOUND 3MI pmol/mg protein/min
LUNG
LIVER
−10
0
10
20
30
1/S [m mol]

against the reciprocal of substrate concentration for lung and liver microsomes is shown in Fig. 6.9.

The Km values were 0.37 and 0.44 mmol for the lung and liver microsomal enzyme systems respectively. The V_{max} values were 100 and 556 pmol covalently bound 3-methylindole/mg microsomal protein/minute for the lung and liver microsomal enzyme systems respectively. Thus the concentration of 3-methylindole required to obtain half the maximal rate of lung microsomal alkylation (0.37 mmol) was less than that required to obtain a similar rate with liver microsomes (0.44 mmol), whereas the magnitude of alkylation of liver microsomes was about five times that of lung microsomes.

6.3 DISCUSSION

The toxic effect of many xenobiotic compounds is based on their conversion to highly reactive metabolites. The metabolites can alkylate or arylate cellular macromolecules and produce tissue damage (Gillette, 1974a, b). It was interesting to find that 3-methylindole becomes covalently bound to tissue proteins in vivo. The highest concentration of covalently bound metabolites occurred in the lung after administration of tritiated 3-methylindole in calves (Table 6.1), suggesting that the pulmonary toxic effect of 3-methylindole may be related to this covalent binding. The fundamental role of reactive metabolites in the pathogenesis of chemically induced lung damage is well documented (Reid et al, 1973; Boyd, 1976; Boyd, 1977; Boyd and Burka, 1978; Boyd, Burka, Wilson and Sasame, 1978; Boyd Statham, Franklin and Mitchell, 1978).

The covalent binding of 3-methylindole metabolite to

microsomal proteins "in vitro" was dependent on temperature, oxygen, reduced nicotinamide adenine dinucleotide phosphate and time (Figs. 6.2 and 6.3). It was inhibited by cytochrome c, SKF-525A (Table 6.2), by carbon dioxide atmosphere and by a carbon monoxide enriched atmosphere (Fig. 6.2). The combined requirements for reduced nicotinamide adenine dinucleotide phosphate and oxygen suggest that a mixed function oxidase enzyme system is involved and that, without prior metabolism, 3-methylindole is not sufficiently reactive to alkylate tissue components. The inhibition of covalent binding by a carbon monoxide atmosphere and cytochrome c implies the participation of cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase respectively as components of the electron transfer chain in the bovine lung microsomal system responsible for the activation of 3-methylindole. This enzyme system has the classical characteristics of a cytochrome P-450 dependent mixed function oxidase.

The components of a cytochrome P-450 dependent mixed function oxidase were identified as cytochrome P-450, reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase, and a lipid component phosphatidylcholine (Lu and Coon, 1968; Strobel, Lu, Heidema and Coon, 1970). The proposed scheme for the transfer of electrons in a cytochrome P-450 dependent mixed function oxidase proceeds as follows: The enzyme, reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase, is first reduced and electrons are transferred to cytochrome P-450. The reduced form of cytochrome P-450 reacts with molecular oxygen in such

a way that one of the oxygen atoms is reduced to water and the other is introduced into the molecule of the foreign compound (Coon, Strobel and Bayer, 1973). The presence of phosphatidylcholine appears to be necessary for the rapid flow of electrons from reduced nicotinamide adenine dinucleotide phosphate to cytochrome P-450 (Strobel, Lu, Heidema and Coon, 1970).

Carbon monoxide inhibits cytochrome P-450 by reacting with the enzyme, when in the reduced form, to form a complex which has no enzymatic activity (Omura and Sato, 1964a, b).

Cytochrome c inhibits cytochrome P-450 dependent mixed function oxidases by competing with cytochrome P-450 for reducing equivalents from reduced nicotinamide adenine dinucleotide phosphate.

Results reported in this present work are consistent with the findings of other workers (Bray and Carlson, 1979a) which showed that piperonyl butoxide (a mixed function oxidase inhibitor) prevented, whereas phenobarbital increased, the pneumotoxic effect of 3-methylindole in goats, suggesting the pulmonary toxic effect is dependent on metabolism of 3-methylindole by mixed function oxidase. Also a preliminary report on the covalent binding of 3-methylindole to microsomal preparations from the lung, liver and kidney of the goat is published (Bray and Carlson, 1979b).

Ultrastructural studies (Huang, Carlson, Bray and Bradley, 1977) have shown that 3-methylindole induced proliferation of the smooth endoplasmic reticulum in type 1 pneumocytes and in non-ciliated bronchiolar lining cells of goat lungs. It is known that the smooth endoplasmic reticulum of cells is the site of mixed function

oxidase enzyme system (Staubli, Hess and Weibel, 1969). Strong nucleophilic substances, viz glutathione, L-cysteine and N-acetyl-L-cysteine inhibited covalent binding of the reactive metabolite of 3-methylindole to microsomal proteins (Table 6.2 and Figs. 6.5 and 6.6, 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 known that the conjugation of glutathione, which is a strong nucleophile, with substrates having a sufficiently strong electrophilic centre may occur spontaneously (non-enzymatically). Also a group of enzymes present in the cytosol of cells, viz glutathione S-transferases can catalyze the conjugation of glutathione with a wide variety of substances. Both types of reactions, the spontaneous and the glutathione S-transferase catalyzed, may occur simultaneously (Testa and Jenner, 1976). Results of this present study indicate that glutathione inhibited covalent binding of 3-methylindole metabolite to microsomal proteins (Fig. 6.5). Addition of the lung cytosol fraction caused an increase in the inhibiting effect of glutathione (Fig. 6.6). These findings suggest that the reaction between glutathione and the reactive metabolite of 3-methylindole 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 3-methylindole metabolites to microsomal proteins was greatest when the cytosol fraction was used with glutathione compared

to cysteine or N-acetyl-L-cysteine (Fig. 6.6). It is known that the substrate specificity of glutathione S-transferases for glutathione is high (Habig, Pabst and Jacoby, 1974). Similar observations were reported by Rollins and Buckpitt (1979) while studying the role of liver cytosol in catalyzing the conjugation of reduced glutathione with a reactive metabolite of acetaminophen. These authors suggested the use of whole cytosol fraction rather than highly purified glutathione S-transferase preparations and concluded that the use of purified enzyme preparations would produce misleading results, since glutathione S-transferase enzymes have broad, overlapping substrate specificities. If transferase enzymes present in the cytosol fractions have different specificities towards the reactive metabolite under investigation, then the use of a few of the highly purified enzymes lacking this specificity might show only minimal activity with the chemically reactive metabolite produced during microsomal metabolism of the foreign compound under investigation.

The role of glutathione is established in the detoxification of foreign compounds which are inherently electrophilic (Smith et al, 1977) and electrophilic metabolites of foreign compounds (Mitchell, Hinson and Nelson, 1976). Results reported in this present study strongly suggest that conjugation with glutathione may be an important pathway for detoxification of the reactive metabolite of 3-methylindole.

The highest concentration of covalently bound metabolites of 3-methylindole occurred in the lung. It is not known whether this is due to a higher rate of reactive metabolite formation in the lung than in the liver, or due to deficiency of the lung in reactive metabolite detoxifying pathways. The first of these possibilities was

investigated *in vitro* by studying and comparing the kinetic parameters of the lung and liver microsomal enzyme systems catalyzing the covalent binding of 3-methylindole. Qualitatively the liver microsomal enzyme system was similar to that of the lung, viz cytochrome P-450 dependent mixed function oxidase (Figs. 6.2 and 6.4).

Quantitatively the Michaelis constant for the lung microsomal system was (0.37 mmol) lower than that for the liver (0.44 mmol) microsomal system. Thus the lung microsomal system has a greater affinity than the liver microsomal system towards 3-methylindole. Boyd, Burka, Wilson and Sasame (1978) have demonstrated that the Michaelis constant for the pulmonary microsomal alkylation by 4-ipomeanol is more than ten-fold lower than for hepatic microsomal pathway, and therefore they suggested that the higher affinity of the lung microsomal system towards 4-ipomeanol may provide a basis for the pulmonary specificity of tissue alkylation and toxicity of the 4-ipomeanol metabolite *in vivo*.

It has been thought that dipyridylium radical cations are necessary intermediates in the mechanism of toxicity of the herbicides paraquat, diquat and morfamquat. Chronic administration of these herbicides causes lung fibrosis, cataracts and renal lesions, respectively, in rats. Baldwin, Pasi, MacGregor and Hine (1975) investigated the hypothesis that the organ selectivities of these herbicides results from differing rates of radical production in different organs by measuring the specific rates of radical cation appearance for these herbicides in homogenates of the lung, kidney and liver of the rat. They found that for each of the three herbicides,

the order of the rates of appearance of the radical was liver > lung > kidney. These findings led Baldwin-coworkers (1975) to discard the hypothesis that the selectively enhanced rates of radical production in the target organs can be the only factor responsible for organ specificities observed with these herbicides.

Results of this present study and of other in vitro studies (Boyd, Burka, Wilson and Sasame, 1978) should be interpreted with caution. In vitro studies using microsomal preparations have the following drawbacks:

(1) They do not take into account the rate of supply of co-factors, such as reduced nicotinamide adenine dinucleotide phosphate required for activity of enzyme systems involved. Different rates of supply of these co-factors in different tissues would affect the rate of generation of cytotoxic reactive metabolites in these tissues.

(2) The rate of alkylation of microsomal proteins, in vitro, reflects not only the rate of reactive metabolite formation but also the rate of reactive metabolite elimination by microsomal enzymes, such as the epoxide hydrase enzymes. Therefore differences in kinetic parameters between the lung and liver microsomal enzyme systems catalyzing the metabolic activation of 3-methylindole reported in this present study and of ipomeanol reported by Boyd, Burka, Wilson and Sasame (1978) could reflect different rates of reactive metabolite formation or alternatively different rates of reactive metabolite elimination by microsomal enzymes.

(3) Microsomal preparations do not take into account non-microsomal detoxifying pathways, such as glutathione S-transferase.

(4) The mammalian lung contains over 20 different specific cell types (Breeze and Wheeldon, 1977). There is considerable evidence that cytotoxic alkylation by reactive metabolite forming pulmonary toxic substances occurs only to certain specific cellular type, viz the Clara cells. These cells are proposed as the site of cytochrome P-450 (Boyd, 1977); therefore microsomal preparations from these Clara cells would be expected to have a higher concentration of foreign compound metabolizing enzymes than those from other cellular types. Microsomal preparations used in this present study and in other studies represent mixed microsomes from all cellular varieties of the lung.

Careful studies may show that the same compound or chemically related compounds causes toxicity in many organs possessing enzyme systems capable of generating chemically reactive metabolites from these compounds. Various furano compounds exhibit different organ specifities, e.g. 4-ipomeanol and 3-methylfuran possess specific pulmonary toxic actions (Boyd and Burka, 1978; Boyd, Statham, Franklin and Mitchell, 1978). Furosemide possesses hepatotoxic and nephrotoxic effects (Jollow and Mitchell, 1973; Mitchell, Potter and Jollow, 1973). The nature of the cytotoxic response may differ from one compound to another. The effects of 4-ipomeanol and furosemide are necrotizing whereas another furano compound aflatoxin B_1 acts as a hepatocarcinogen (Wogan and Newberne, 1967; Carnaghan, 1967). In the case of indolic compounds, it has been established that 3-methylindole possess pulmonary toxic effect (Pirie et al, 1976) and there is some evidence to suggest that it also possesses hepatotoxic effect. Cattle affected with pneumotoxicity after oral

doses of L-tryptophan displayed a reduced concentration of tryptophan pyrrolase in their liver. This has been demonstrated by Johnson and Dyer (1966) whose study was not designed to investigate the hepatotoxic effects of 3-methylindole. Although not able to be demonstrated by histopathology the reduction in tryptophan pyrrolase activity can be considered as a hepatotoxic response which is probably due to alkylation of this enzyme by the reactive metabolites of 3-methylindole. This point merits further investigation. Another indolic compound, indole, has recently been shown to possess nephrotoxic effect and it has been suggested that release of haemoglobin from erythrocytes haemolyzed by indole is responsible for the renal tubular necrosis caused by indole (Hammond, Carlson and Breeze, 1980). The nephrotoxic effect of furosemide is thought to be due to chemically reactive metabolite formation (Mitchell, Potter and Jollow, 1973). Similarly indole should be evaluated as a reactive metabolite forming nephrotoxic agent.

The particular organ damaged by a foreign compound may be the result of the balance between two factors. The rate of formation of the reactive metabolite and the rate of its elimination in that organ. The specific injury provoked is probably a consequence of the affinity of the reactive metabolite for a particular cellular macromolecule.

CHAPTER 7

PHARMACOLOGICAL MODULATION OF THE PULMONARY TOXIC EFFECT OF 3-METHYLINDOLE

7.1 - INTRODUCTION

A) - The following approaches can be exploited to decrease the pulmonary toxic effect of 3-methylindole.

7.1.1 - Possible induction of tolerance to 3-methylindole

It has been shown that pretreatment of rats with sublethal doses of 4-ipomeanol (10 mg/kg) renders rats tolerant to normally lethal doses (> 30 mg/kg) of this pulmonary toxic compound (Boyd, Burka, Osborne and Wilson, 1975). At least two factors contribute to the development of tolerance to chemically-induced lung damage:

1) Decreased rate of generation of reactive metabolites

Covalent binding of radiolabelled 4-ipomeanol to lung tissues of tolerant (4-ipomeanol pretreated) rats was significantly less than that to normal rats. In vitro, there was a significant decrease in enzymatic covalent binding of 4-ipomeanol to lung microsomes from tolerant rats. Thus it has been suggested that tolerance results from a decrease in lung mixed function oxidase activity produced by the pretreatment dose of 4-ipomeanol; therefore, on subsequent challenge with larger doses of the pneumotoxic compound, reactive metabolites are not formed in a concentration sufficiently high to produce lung lesions (Boyd, Burka, Osborne and Wilson, 1975). The decrease in mixed function oxidase activity is probably due to alkylation of microsomal enzymes be reactive metabolites. Reactive metabolite-enzyme conjugates possess no enzymatic activity. It has been shown that 3-methylindole induces rapid tolerance (tachyphylaxis) in calves (Atkinson et al, 1977).

2) Enhanced biochemical defence mechanisms

Recent studies have shown that antioxidant defence systems of the lung, viz, reduced glutathione and glutathione peroxidase, catalase and superoxide dimutase enzymes play a crucial role in protecting the lung against oxidant-induced injury. Pretreatments which enhance activity of these defence systers, protect the lung against oxygen-induced lung damage (Yam and Roberts, 1979). Also enhancement of these defence systems appears to be responsible for the development of tolerance to chemically-induced lung damage. There is a close temporal relationship between the development of tolerance to chemically-induced lung damage and certain histological and biochemical changes. Histological changes are represented by proliferation of type 2 pneumocytes which occur within 24 to 48 hours after exposure of the lung to the pulmonary toxic agent (Fairchild, 1967; Smith, Winter and Wheelis, 1973) and which can be induced by a variety of pulmonary toxic agents such as ozone, nitrogen dioxide, high tension of oxygen, bleomycin, thiourea, oleic acid, monocrotaline and paraquat (Yuen and Sherwin, 1971; Bowden and Adamson, 1971; Kilburn, 1972). Biochemical changes are represented by an increase in the concentrations of reduced glutathione, glutathione reductase and glutathione peroxidase in the lung 24 to 48 hours after initiation of exposure to these agents (Chow and Tappel, 1973; Cross, De Lucia and Mustafa, 1974) which is about the length of time required for the development of tolerance (Fairchild, 1967; Smith, Winter and Wheelis, 1973). This temporal relationship between development of tolerance to type 2 pneumocyte hyperplasia and the increase in the antioxidant defence systems of the lung led Cross (1974) to hypothesize that type

2 pneumocytes are involved in antioxidant defence of the lung. Induction of type 2 pneumocyte hyperplasia would decrease the susceptibility of the lung parenchyma to a wide variety of pulmonary toxic agents.

It has been shown that 3-methylindole administration induces pneumocyte type 2 hyperplasia in cattle (Pirie et al, 1976) and in goats (Huang et al, 1977). It is not known whether this is associated with development of tolerance to 3-methylindole or not.

7.1.2 - Modifying metabolic pathways involved in the generation of the reactive metabolite of 3-methylindole

Studies presented in Chapter 6 indicate that mixed function oxidase is involved in the generation of the 3-methylindole reactive metabolite in cattle. It would be anticipated that inhibition and induction of microsomal mixed function oxidase will prevent and enhance the 3-methylindole-induced lung lesions, respectively. In vivo studies have shown that mixed function oxidase inducers and inhibitors increase and decrease, respectively, the severity of the pneumotoxic effect of 3-methylindole in goats (Gray and Carlson, 1979a).

7.1.3 - Modifying metabolic pathways involved in the detoxification of the reactive metabolite of 3-methylindole

Studies presented in Chapter 6 indicate that the reactive metabolite of 3-methylindole can be detoxified by becoming conjugated with glutathione. Thus modifying the level of glutathione by administration of compounds such as diethylmaleate which depletes

glutathione or L-cysteine which increases glutathione would increase and decrease, respectively, the severity of the 3-methylindole-induced lung lesions.

Studies on 4-ipomeanol have shown that treatment of rats with diethylmaleate increases the severity of 4-ipomeanol-induced lung lesions in rats (Boyd and Eurka, 1978).

7.1.4 - Failure of antagonists to known mediators of anaphylaxis to protect cattle against the 3-methylindole-induced pneumotoxicity

Hammond, Carlson, Breeze and Selman (1979) investigated the possible use of a group of compounds known to protect cattle to some extent against the effects of anaphylaxis in the treatment of 3-methylindole-induced pulmonary damage in cattle. An oral dose of 3-methylindole (0.2 g/kg) was administered to groups (two animals each) of cattle which had been pretreated with acetylsalicylic acid (100 mg/kg, orally every 12 hours beginning 24 hours before the 3-methylindole dose), mepyramine maleate (5 mg/kg, intramuscularly every 12 hours, beginning 20 minutes before the 3-methylindole dose), sodium meclofenamate (20 mg/kg, intramuscularly every 24 hours, beginning 1 hour before the 3-methylindole dose), diethylcarbamazine citrate (1 ml 40% w/v solution/20 kg, intramuscularly every 24 hours, beginning 24 hours before the 3-methylindole dose) or betamethasone (20 mg/50 kg, intramuscularly every 12 hours, beginning 12 hours before the 3-methylindole dose) or to control cattle. The authors of this study stated that none of the drugs appeared to influence significantly the clinical course of the disease.

B) - The pneumotoxic effect of 3-methylindole in sheep

Bradley, Carlson and Dickinson (1978) administered 3-methylindole (intraruminally) to two groups of sheep (four animals each). One group was given a dose of 0.6 g/kg. This treatment resulted in the death of all treated sheep. The other group was given a dose of 0.2 g/kg which resulted in the death of only one animal. All animals, in the two groups, showed clinical signs of acute respiratory distress. Post-mortem examination showed that the lungs of these animals were inflated, dark red and had a turgid texture. Microscopically alveolar and interstitial oedema were the predominant changes observed. It was reported that a consistent change, observed in terminal bronchioles of all sheep that died, was that lining epithelia of terminal bronchioles were irregularly swollen and many cells were necrotic and detached from the basement membrane.

7.2 EXPERIMENTAL

7.2.1 - Introduction

In vitro studies reported in Chapter 6 have shown that the chemically reactive metabolite of 3-methylindole combines preferentially with glutathione. Thus pretreatments that alter the availability of glutathione would also alter the severity of the pneumotoxic effect of 3-methylindole in this series of experiments:

1) The effect of depleting glutathione on the 3-methylindole-induced pulmonary damage is investigated. Diethylmaleate was used to deplete glutathione. It has been shown that administration of this compound depletes glutathione in many organs including the lung (Boyland and

and Chasseaud, 1970; Richardson and Murphy, 1975). This is due to rapid enzyme-catalyzed conjugation of diethylmaleate with glutathione. 2) The effect of L-cysteine, a precursor of glutathione, on the 3-methylindole-induced pneumotoxicity was also investigated.

Sheep were used as the experimental animal model. The pulmonary toxic response was assessed by:

a) Observing animals after 3-methylindole administration;

b) Determining the time of death of sheep. It has been shown that the cause of death of sheep after 3-methylindole administration is due to pulmonary damage;

c) Determining the degree of pulmonary oedema. This was done by two methods:

(i) Determining the lung weight as percentage of total body weight;

(ii) Determining the ratio $\frac{\text{wet lung tissue weight}}{\text{dry lung tissue weight}}$. 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 porcelain dish. Wet tissue weight was determined and tissue left to dry to a constant weight in an oven at $65^{o}C$;

d) Postmortem and histopathological examination: The lungs of animals were examined after death. Portions of tissue were taken from the lungs and bronchial tree, fixed, embedded in paraffin wax and processed by standard methods.

7.2.2 - Materials

Diethylmaleate was obtained from B.D.H. Chemicals Ltd., Poole, Dorset, England. L-cysteine was obtained from Koch-Light Ltd., Colnbrook, Bucks, England. Other chemicals were obtained from commercial sources as described previously.

7.2.3 - Animals

Male, castrated, black-face cross breed sheep (38-55 kg body weight). Animals were maintained on hay and concentrates.

7.2.4 - Preparation of solutions for injection

3-Methylindole solution was prepared in 10% "Cremophor-EL" in physiological saline solution. The concentration of 3-methylindole was 30 mg/ml. L-cysteine (100 mg/ml) was dissolved in physiological saline solution, immediately before administration. Diethylmaleate was administered without dilution.

7.2.5 - Route of administration

All compounds were administered by intraperitoneal injection.

7.2.6 - Effect of diethylmaleate on the 3-methylindole-induced pulmonary toxicity in sheep

In this experiment five groups of sheep were used:

Group I: Four sheep (designated 1 to 4) each was dosed with 3-methylindole (0.2 g/kg).

Group II: Two sheep (designated 5 and 6) each was given a dose of 3-methylindole (0.2 g/kg) and a total amount of 0.6 ml diethylmaleate/kg bodyweight, divided into two equal doses, the first dose 30 minutes

before 3-methylindole administration and the second dose 30 minutes after 3-methylindole administration.

Group III: Five sheep (designated 7 to 11) each was given a dose of diethylmaleate (0.15 ml/kg) and a dose of 3-methylindole (0.2 g/kg) 30 minutes after diethylmaleate administration.

Group IV: Two sheep (designated C1 and C2) were given diethylmaleate alone (0.6 ml/kg) as described for Group II.

Group V: Two sheep (designated C3 and C4) were given diethylmaleate (0.15 ml/kg) alone.

Results

The experiment and results are summarized in Table 7.1.

Group I

All sheep showed depression, nasal discharge and tachypnoea within 10 to 20 minutes after 3-methylindole administration. As respiratory distress progressed animals showed difficult breathing and a decrease in respiratory rate. This became obvious about one hour after 3-methylindole administration. Respiratory distress was maximal at 2 hours with an audible expiratory grunt.

Animals improved over the next 10 hours and became apparently normal by 72 hours.

One animal (No. 4) was killed after 72 hours and two other animals (Nos. 2 and 3) were killed after 7 days. Postmortem examination of these animals showed circumscribed patches (1-2 cm in diameter) of subpleural congestion scattered all over the lungs, particularly the diaphragmatic surface. No other abnormalities were detected. Microscopic examination of tissue sections from the lungs of these

sheep (Nos. 2, 3 and 4) showed that histopathological changes were mild and focal in distribution (Fig. 7.1). Some areas were normal, whereas in other areas alveolar septa were thickened due to oedema and infiltration with mononucleated cells (Fig. 7.2). The epithelial lining of the bronchi was normal and their lumen contained variable numbers of mononucleated cells whose cytoplasm had a foamy appearance and neutrophils, mixed with cell debris and acidophilic material (Fig. 7.3).

Group II

Animals showed within 10-20 minutes muscle trembling (particularly muscles of flank region), depression and nasal discharges with severe and progressive respiratory distress. At one hour after 3-methylindole administration breathing was irregular and difficult with an audible expiratory grunt and profuse nasal discharges. About 4 hours all animals assumed lateral recumbency with extended neck, frothing from the mouth, and died at 6-10 hours.

Postmortem examination showed that the lungs were dark red, turgid in texture, with streaks of subpleural haemorrhages. They did not collapse completely. The trachea and bronchi were congested and had petechial haemorrhages. The bronchial tree was dry in one animal (No. 6), but filled with white froth of oedema fluid in the other animal (No. 5).

In both animals histopathological changes were severe and diffused. Severe congestion and foci of recent haemorrhages were observed. The alveoli contained acidophilic deposits and mono-nucleated cells and alveolar septa were thick. Epithelial lining of bronchi was normal and their lumen contained considerable numbers of

cells (Fig. 7.5). In some cases the shape of these cells was clear and they had columnar, cuboidal or rounded appearance, pyknotic nuclei and some of them had cilia (Fig. 7.6). In other cases these cells tended to form flakes. The epithelial cells lining the bronchioles had pyknotic nuclei and were seen in different degrees of detachment from the basement membrane. Some bronchioles had few detached cells, whereas the lining of others was completely denuded.

Group III

Within 10-25 minutes, animals showed severe depression and nasal discharges. Difficult irregular breathing with an audible expiratory grunt was obvious at 2 hours. By 4 to 5 hours, animals assumed lateral recumbancy with extended head. Animals died 7-24 hours after 3-methylindole administration.

Postmortem examination showed that the lungs of these animals were dark red with streaks of subpleural haemorrhages. The trachea and bronchial tree were congested and there were submucosal petechial haemorrhages. A white froth was observed in the bronchial tree of one animal (No. 15).

Histopathological changes were similar to those reported for Group II. The changes in alveolar morphology were either severe in some foci or mild in others. The latter foci were small and scattered and there were changes in other areas of the same histological section which were severe (Fig. 7.7). Changes in bronchial and bronchiolar morphology were similar to those described for Group II.

Group IV

These animals showed severe depression and muscle trembling

(particularly muscles of the flank region) 1 to 2 hours after administration of the first dose of diethylmaleate. One animal died at 24 hours and the other animal was in poor condition when killed at 48 hours.

Group V

No clinical signs were detected. Animals were in a good condition and maintained good appetite.

7.2.6 - Effect of L-cysteine on the 3-methylindole-induced pulmonary toxicity in sheep

In this experiment three groups of sheep were used:

Group VI: Four sheep (designated 12 to 15) received 0.3 g/kg 3-methylindole.

Group VII: Four sheep (designated 16 to 19) were each given a dose of L-cysteine 0.3 g/kg followed by a dose of 3-methylindole 0.3 g/kg 60 minutes after L-cysteine administration.

Group VIII: Two sheep (designated C5 and C6) were each given a dose of L-cysteine (0.3 g/kg).

Results

The experiment and results are summarized in Table 7.1.

Group VI

Three sheep (Nos. 12 to 14) showed severe depression, nasal discharges and trembling of the muscles of the flank. This became evident 10-20 minutes after 3-methylindole administration. By two hours animals assumed lateral recumbancy with difficult irregular breathing and an audible expiratory grunt. Only one animal (No. 12) showed profuse frothing from the mouth. The three

sheep died two to seven hours after 3-methylindole administration. The remaining animal (No. 15) showed signs similar to those reported in the other three animals, but moderate in severity and delayed in onset. At eight hours respiratory distress was severe, the animal assumed lateral recumbancy and died at 10 hours.

Lungs of animals 13 and 14 were severely congested, did not show complete collapse and were turgid. The trachea and bronchial tree were congested and showed petechial haemorrhages. The bronchial tree of animal No. 13 was filled with a white froth.

Lungs of animal No. 15 showed scattered areas of congestion and slight congestion of the trachea and bronchi. No other abnormalities were detected on gross examination of the lungs of this animal. Histopathological examination showed that changes in alveolar morphology varied in severity in different areas of the same tissue section and were moderate to severe in animals 13 and 14 and mild to severe in animal 15. These changes and changes in bronchioles and bronchi were similar to those described for Group II (Figs. 7.6 and 7.9).

Group VII

Two animals (Nos. 16 and 17) developed bloat after L-cysteine administration. The condition of these two sheep continued to deteriorate after 3-methylindole administration with increasing bloat, progressive respiratory distress (nasal secretions and laboured breathing). By 10 hours both animals became recumbent. One of the animals died at 18 hours and the other at 20 hours.

The other two sheep (Nos. 18 and 19) showed little amount of nasal discharges, mild tachypnoea and depression during the first

hour after 3-methylindole administration. Then respiratory rate decreased with difficulty in breathing; this was evident at five hours after 3-methylindole administration. At eight hours the clinical condition of the animals was improving. They started to be more alert, with disappearance of nasal secretions and breathing appeared normal. At 48 hours both animals were apparently normal.

Postmortem examination, which was conducted four days after 3-methylindole administration, found that the lungs of animals Nos. 18 and 19 were congested and turgid. The trachea and bronchi were congested and petechial haemorrhages were observed. These changes were moderate in the lungs of animal No. 18, but mild in the lungs of animal No. 19.

Histopathological changes were focal in distribution and were mild in animal No. 18 and moderate in animal No. 19. These changes were characterized by thickening of the alveolar septa and alveoli contained various amounts of fibrillar and granular acidophilic deposits. Bronchi and bronchioles contained various numbers of macrophages, neutrophils and cell debris.

Group VIII

Slight tympany was observed in one animal about two hours after L-cysteine administration, which lessened during the next six hours. The other animal did not show any sign of abnormality. Both animals maintained good appetite and were in a good condition during a period of observation of 72 hours.

TABLE 7.1 EFFECT OF 3-METHYLINDOLE ON NORMAL SHEEP AND ON SHEEP PRETREATED WITH DIETHYLMALEATE OR L-CYSTEINE

Animal Groups and Numbers	Treatment	Weight (kg)	Time of Death	Lung weight (% of body weight)	Wet lung tissue weight / Dry lung tissue weight
Group I:	3MI				
1	(0.2 g/kg)	38	S	N.D.	N.D.
2		40	S, K, 7 days	1.5	4.9
3		40	S, K, 7 days	1.3	5.1
4		45	S, K, 72 hours	1.5	4.8
				(1.4 ± 0.1)	(4.9 ± 0.1)
Group II:	3MI				
5	(0.2 g/kg)	40	D - 10 hours	2.5	7.3
6	+	42	D - 6 hours	2.0	6.4
	DM				
	(0.6 ml/kg)			(2.3 ± 0.2)	(6.9 ± 0.5)
Group III:					
7	3MI	45	D - 18 hours	N.D.	N.D.
8	(0.2 g/kg)	43	D - 20 hours	N.D.	N.D.
9	+	45	D - 7 hours	1.8	5.3
10	DM	50	D - 15 hours	1.4	5.1
11	(0.15 ml/kg)	40	D - 8 hours	1.5	5.4
				(1.6 ± 0.2)	(5.3 ± 0.1)

TABLE 7.1 (Contd)

Animal Groups and Numbers	Treatment	Weight (kg)	Time of Death	Lung weight (% of body weight)	Wet lung tissue weight / Dry lung tissue weight
Group VI:	3MI				
12	(0.3 g/kg)	45	D - 2 hours	N.D.	N.D.
13		43	D - 7 hours	1.8	7.5
14		55	D - 5 hours	2.0	5.8
15		45	D - 10 hours	1.3	5.1
				(1.7 ± 0.3)	(6.1 ± 1.0)
Group VII:	3MI				
16	(0.3 g/kg)	55	D - 18 hours	N.D.	N.D.
17	+	40	D - 20 hours	N.D.	N.D.
18	L-cyst. (0.3 g/kg)	45	S, K - 4 days	1.8	4.6
19		43	S, K - 4 days	1.1	4.9
				(1.5 ± 0.6)	(4.7 ± 0.1)
Group IV:	DM				
C1	(0.6 ml/kg)	40	D - 24 hours	N.D.	N.D.
C2		43	S, K - 48 hours	N.D.	N.D.

TABLE 7.1 (Contd)

Animal Groups and Numbers	Treatment	Weight (kg)	Time of Death	Lung weight (% of body weight)	Wet lung tissue weight / Dry lung tissue weight
Group V:	DM				
C3	(0.15 ml/kg)	44	S	N.D.	N.D.
C4		43	S	N.D.	N.D.
Group VIII:	L-cyst				
C5	(0.3 g/kg)	40	S	N.D.	N.D.
C6		42	S	N.D.	N.D.

3MI = 3-methylindole

DM = Diethylmaleate

L-cyst = L-cysteine

S, K = Survived, and killed by pentobarbitone euthanasia

D = Died

( ) = Mean ± S.E.M.

N.D. = Not determined

Fig. 7.1 Lung of sheep No. 2 which was killed seven days after receiving a dose of 3-methylindole (0.2 g/kg). Changes were mild and focal in distribution. (H & E stain; × 35)

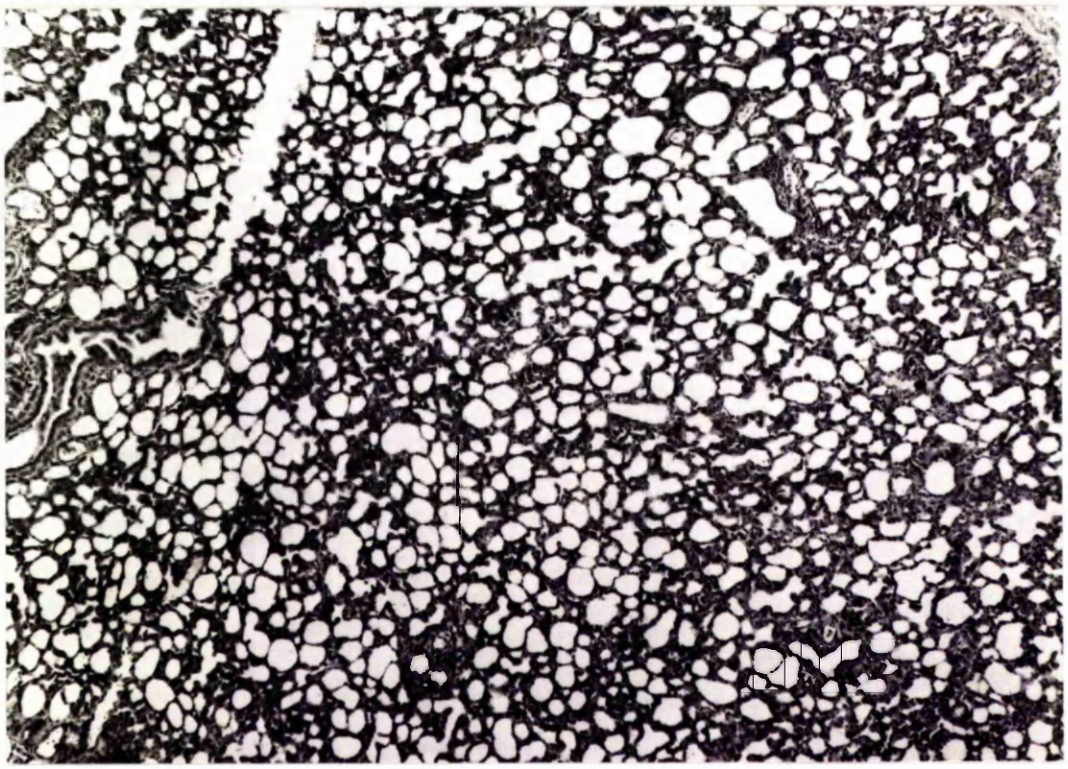


Fig. 7.2 High magnification of Fig. 7.1. Alveolar septa of some alveoli were normal, whereas in the other alveoli it was slightly thickened due to oedema and infiltration with mononucleated cells.
(H & E stain; × 250)

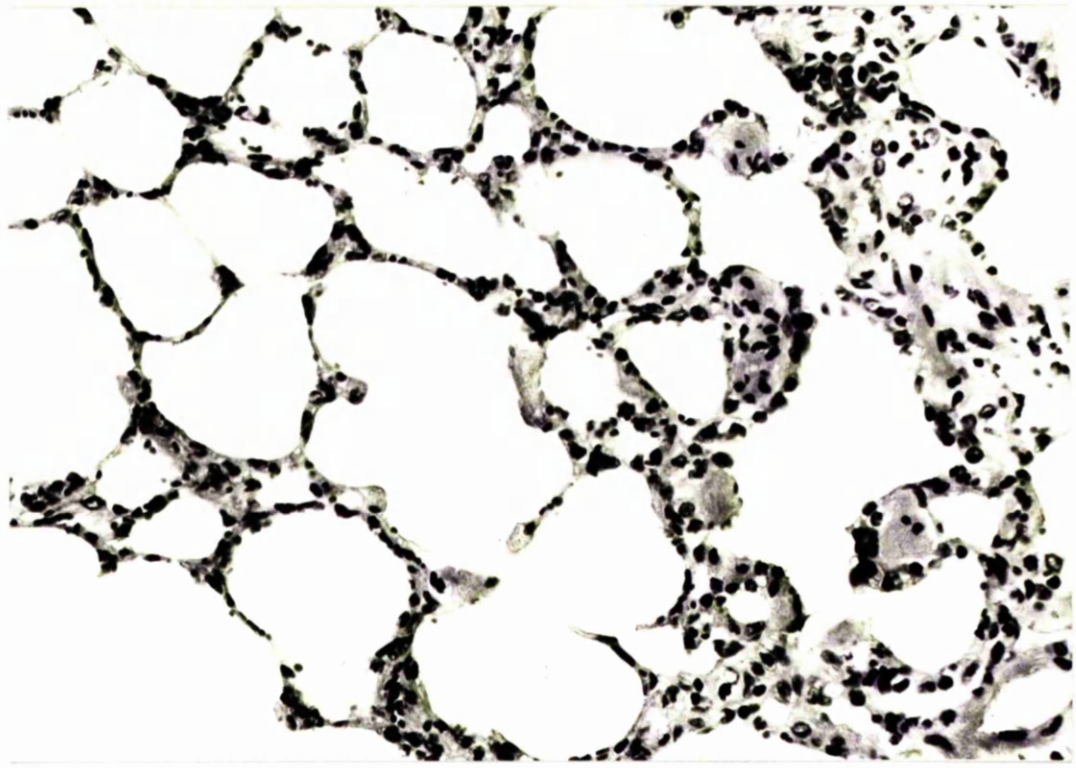


Fig. 7.3 The lung of the same animal as in Fig. 7.1. The bronchial tree contained variable numbers of macrophages, neutrophils, mixed with cell debris and acidophilic material.
(H & E stain; × 110)

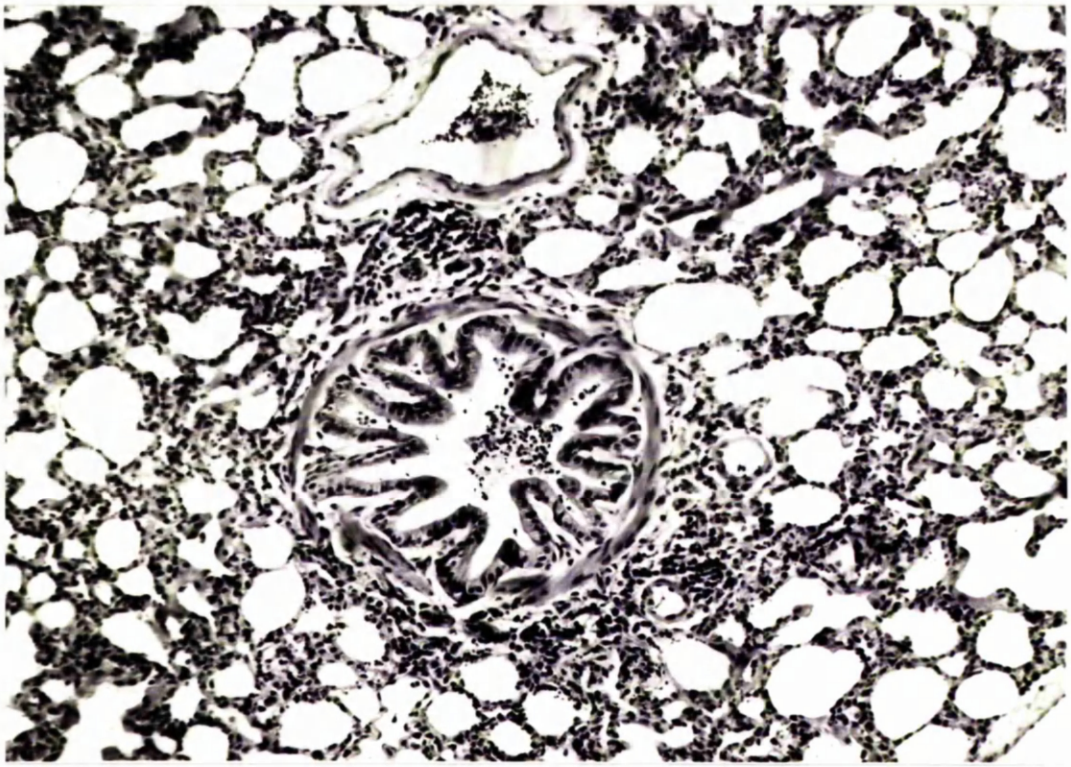


Fig. 7.4 Lung of sheep No. 6, which died after receiving doses of 3-methylindole (0.2 g/kg) and diethylmaleate (0.6 ml/kg). The epithelial lining of the bronchioles had pyknotic nuclei and were seen detached from the basement membrane.
(H & E stain; × 250)

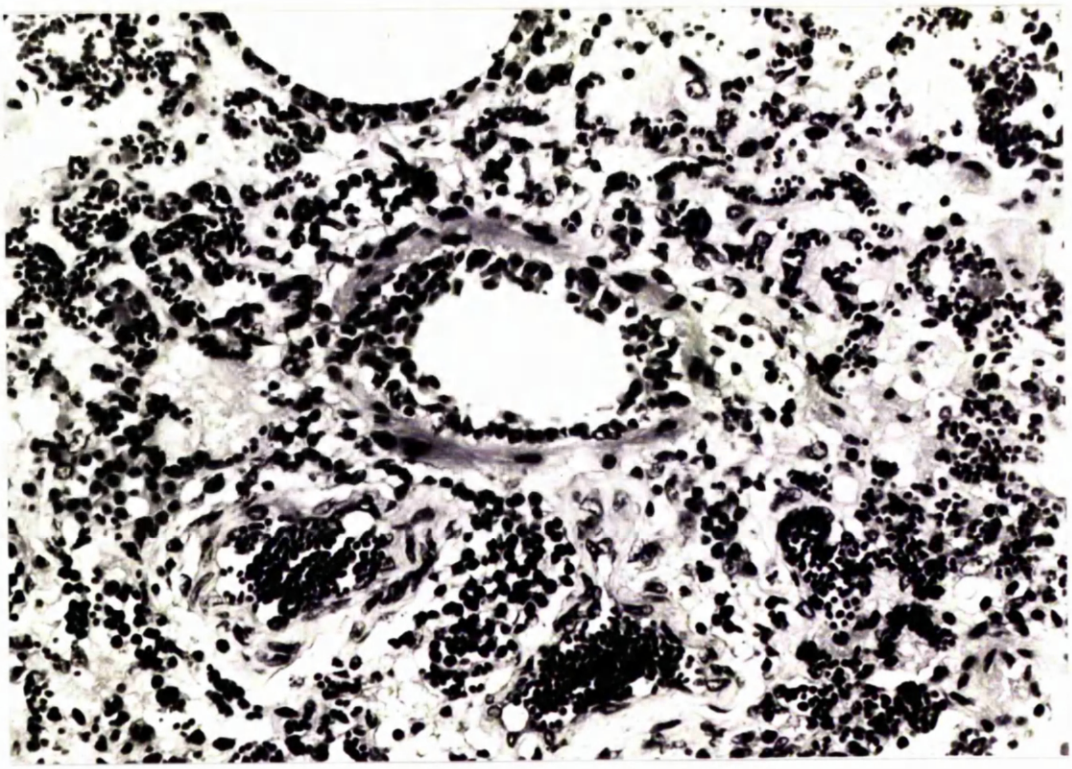


Fig. 7.5 The lung of the same animal as in Fig. 7.4. A bronchus with normal epithelial lining and its lumen containing exfoliated epithelial cells. (H & E stain; × 35)

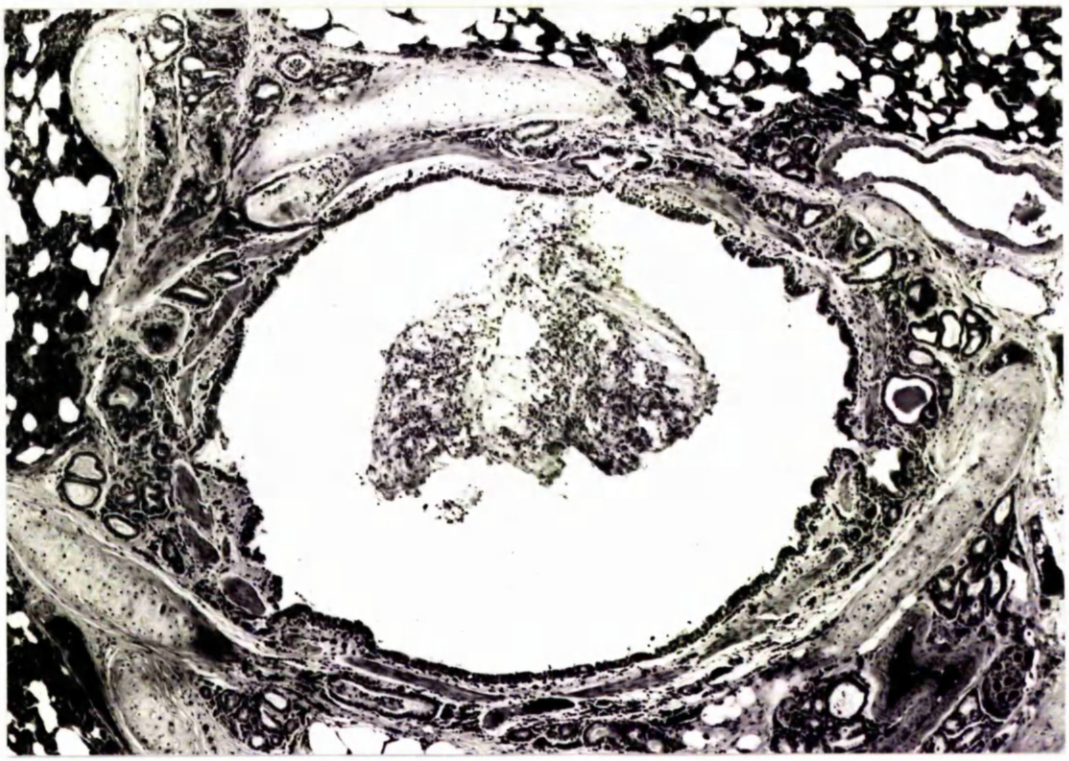


Fig. 7.6 High magnification of cells in the lumen of the bronchus in Fig. 7.5. The cells had columnar, cuboidal or rounded appearance and pyknotic nuclei. Cilia can be seen in some of them. (H & E stain; × 1300)

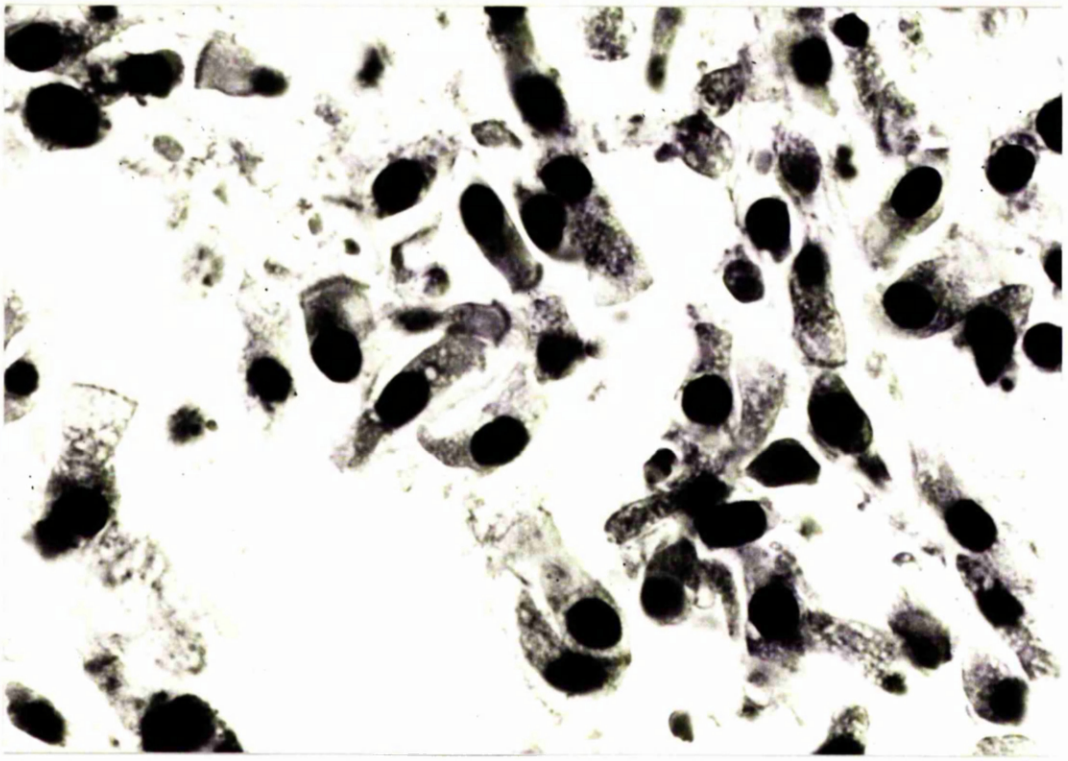


Fig. 7.7 Lung of sheep No. 9, which died after receiving doses of 3-methylindole (0.2 g/kg) and diethylmaleate (0.15 ml/kg). Congestion, oedema and bronchiolar epithelial necrosis can be seen. Changes in alveolar morphology were focal.
(H & E stain; × 35)

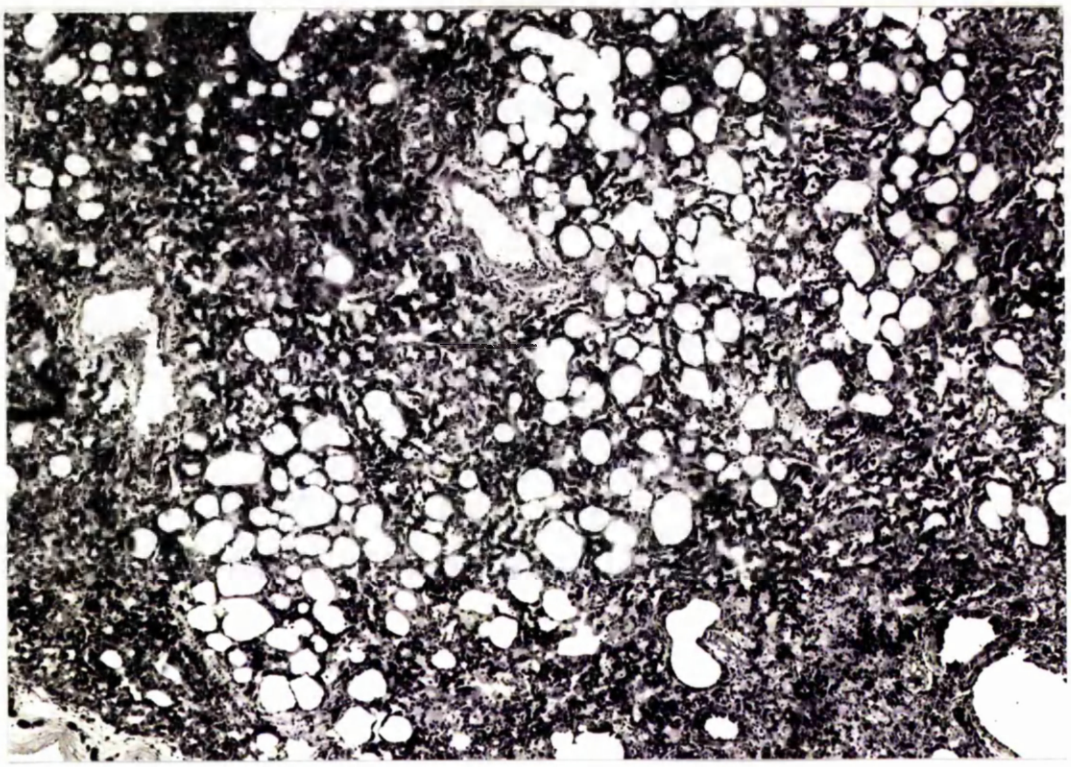


Fig. 7.8 Lung of animal No. 14 which died after receiving a dose of 3-methylindole (0.3 g/kg). Congestion, oedema and bronchiolar epithelial necrosis can be seen. (H & E stain; × 110)

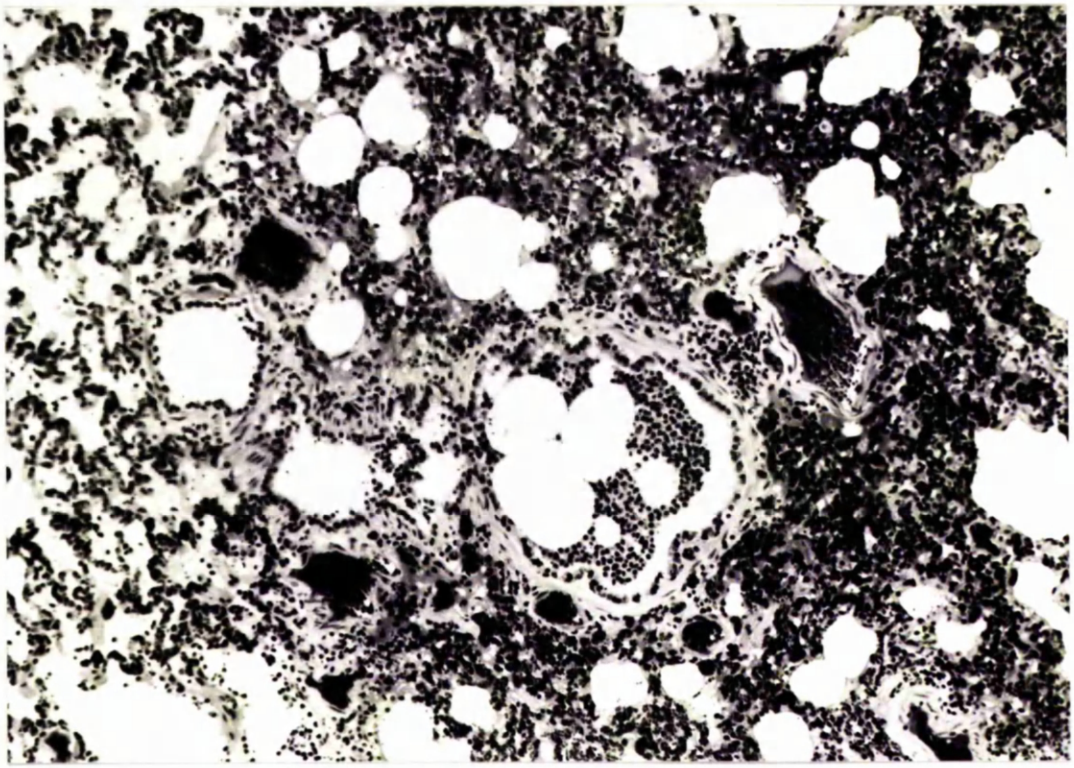
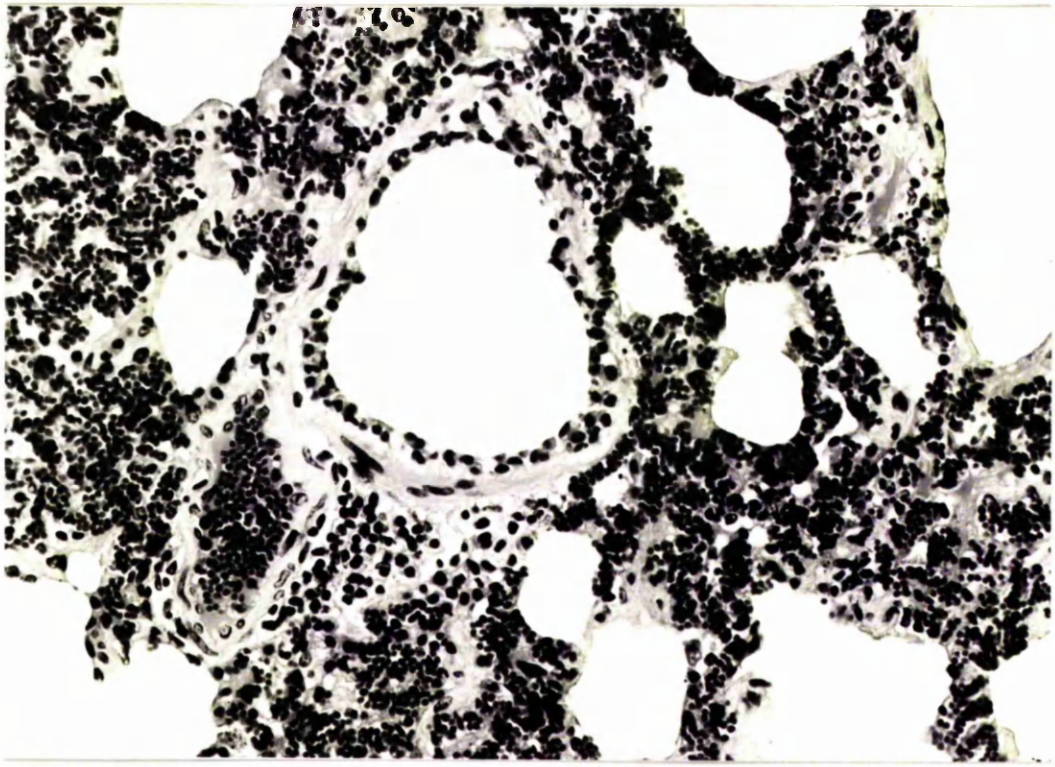


Fig. 7.9 Lung of animal No. 15 which died after receiving a dose of 3-methylindole (0.3 g/kg). Congestion and bronchiolar epithelial necrosis can be seen.
(H & E stain; × 250)



7.3 - DISCUSSION

In this series of experiments, pretreatments which are known to deplete and increase cellular glutathione, respectively, appeared to increase and decrease, respectively, the pneumotoxic effect of 3-methylindole in sheep.

Administration of 3-methylindole (0.2 g/kg) alone did not cause death in any of four treated sheep. In contrast pretreatment of sheep with diethylmaleate (0.15 ml/kg) 30 minutes before administration of the same dose of 3-methylindole (0.2 g/kg) increased the severity of respiratory distress and pathological changes in the lung leading to death of all treated animals (five sheep). It is known that diethylmaleate depletes glutathione in many organs including the lung (Boyland and Chasseaud, 1970; Richardson and Murphy, 1975). The dose range and effects of diethylmaleate in sheep are not known. In this present study two doses of this compound were used. The larger dose (0.6 ml/kg) is equal to that used by Boyd and Burka (1978) to deplete lung glutathione in rats, and was found to cause toxic effects in sheep. The smaller dose (0.15 ml/kg) did not cause any observable toxic symptoms in two sheep.

A larger dose of 3-methylindole (0.3 g/kg) caused death in all (four) treated sheep within 10 hours after 3-methylindole administration. Pretreatment of sheep with L-cysteine (0.3 g/kg) decreased the severity of symptoms, pathological changes in the lung, and rate of mortality following administration of the same dose (0.3 g/kg) of 3-methylindole. Only two sheep died when this dose of 3-methylindole was administered to four L-cysteine pretreated

sheep. The death of these two sheep was delayed (18 and 20 hours) and was at least in part due to ruminal tympany which would certainly increase the respiratory embarrassment resulting from 3-methylindole administration. The protective effect of L-cysteine can be attributed to:

1) Increased availability of glutathione offering alternative nucleophilic sites for covalent binding with the 3-methylindole chemically reactive, electrophilic, metabolite.

2) L-cysteine itself can act as a nucleophilic substrate for covalent binding with the 3-methylindole reactive metabolite.

Results of experiments in Chapter 6 show that nucleophilic substances L-cysteine, N-acetyl-L-cysteine and glutathione prevent the alkylation of microsomal proteins by the 3-methylindole reactive metabolite (in vitro), suggesting that glutathione and glutathione S-transferases are important in the detoxication of the 3-methylindole reactive metabolite. Results of in vivo studies reported in this chapter are in excellent agreement with this conclusion and confirm the usefulness of the "in vitro" strategies, adopted in Chapter 6, in probing the mechanism of action of pulmonary toxic substances.

The possible role of glutathione in protecting lung parenchyma against chemically reactive metabolite-induced lung injury has previously been investigated by other workers (Boyd and Burka, 1978) who have shown that pretreatment of rats with diethylmaleate increases both the alkylation of lung tissue and severity of lung lesions caused by 4-ipomeanol. However, the possible use of precursors of glutathione and other nucleophilic agents in the

prevention of this type of lung damage has not previously been investigated. Results of this work have shown that L-cysteine, and possibly other nucleophilic and thiol compounds, can be used for the treatment of 3-methylindole toxicity. The time of administration and bioavailability of the administered nucleophilic compound would be critical for effectiveness of treatment of this type of toxicity. Administration of these compounds would have to be initiated as soon as possible after the onset of the first clinical signs of respiratory distress. Treatment would be of doubtful value once the disease has progressed.

It has been shown that cysteine (Strubelt, Siegers and Schutt, 1974) and N-acetyl-L-cysteine (Piperno and Berssenbruegge, 1976) reduce the hepatotoxicity of acetaminophen (paracetamol) in animals. This method of treatment is now the standard method of treatment of overdosage with acetaminophen in humans (Gilman, Goodman and Gilman, 1980). Histopathological studies reported here and those by other investigators (Bradley et al, 1978) have shown that necrosis of bronchiolar epithelium is a reproducible and consistent effect of lethal doses of 3-methylindole. 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 and between different animals which had received the same treatment. Similarly pneumotoxic furano compounds and halobenzenes produced consistent changes in bronchiolar epithelium, whereas the

changes in alveolar morphology were less consistent (Reid et al, 1973; Boyd, 1977; Boyd and Burka, 1978; Boyd, Statham, Franklin and Mitchell, 1978). It has been suggested that the Clara cells are the site of cytochrome P-450-dependent mixed function oxidases in the lung. Therefore these cells are the target for reactive metabolite forming pneumotoxic compounds, whose reactive metabolites are formed by a cytochrome P-450-dependent mechanism. Presence of the Clara cells in large numbers in the epithelium of bronchioles renders these bronchioles more susceptible to the effect of these pneumotoxic compounds (Boyd, 1977). Oedema and alteration in alveolar morphology are changes secondary to bronchiolar injury.

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Much of the available information on the aetiology of "fog fever" (acute bovine pulmonary emphysema) including the results of this work is in favour of the hypothesis that the disease is caused by excessive production of 3-methylindole by ruminal microorganisms, from non-pneumotoxic indolic substrates in grass. It appears that at least three factors can contribute to excessive production of 3-methylindole in the rumen under natural conditions: Presence of sufficient amount of indolic precursors (e.g. L-tryptophan and indoleacetic acid) convertible to 3-methylindole in grass, together with the enhanced conversion of these indolic precursors to 3-methylindole by ruminal microorganisms and absence of certain substances (e.g. carbohydrates) which can inhibit the conversion of these indolic precursors to 3-methylindole.

Failure to identify significant differences in the concentration of L-tryptophan between grass-inducing the disease and normal pasture (Mackenzie et al, 1975; Selman et al, 1977), together with observations showing that there is a considerable variation in the incidence, between different herds, and severity, between different individuals of the same herd, of respiratory distress after exposure of cattle to the same inducing agent (whether a change to better grazing or orally administered L-tryptophan) (Dickinson and Carlson, 1978; Carlson and Dickinson, 1978) suggested that it is not only the excessive intake of L-tryptophan which causes the disease but that other factors must contribute. Results of experimental work presented

in this thesis strongly suggest that the enhanced conversion of L-tryptophan, and probably other indolic compounds, to 3-methylindole is a major factor necessary for occurrence of the disease. This study has also established that normal constituents of grass (carbohydrates) can inhibit the production of 3-methylindole. Thus this result lends weight to the hypothesis that the disease occurs in animals grazing on grass with depleted carbohydrate stores (as a result of use of ammonia fertilizers) (Selman et al, 1976).

The disease usually occurs after introduction of hungry adult cattle to highly digestible good quality lush pasture. The role of this sudden change to better grazing in the disease process is not known. However it can cause a change in ruminal microorganisms which would influence the metabolism of indolic compounds in the rumen. The use of antibacterial agents and substances known to have antimetabolic effects to inhibit the conversion of indolic precursors to 3-methylindole has been suggested (Hammond, Carlson, Breeze and Selman, 1979). From this work a number of antibacterial agents have shown promise as therapeutic agents by altering the microbial flora of the rumen and thereby altering the progress of the disease. However, some of these may prove toxic in this situation. Monensin, for example, which has been suggested as a possible prophylactic drug for fog fever, while reducing the production of 3-methylindole, enhanced the production of indole which resulted in renal lesions (Hammond, Carlson and Breeze, 1980).

The pneumotoxic effect of 3-methylindole appears to be due to its metabolic activation to a chemically reactive metabolite which binds to cellular macromolecules of the lung causing cellular injury.

In support of this hypothesis, the highest concentration of covalently bound metabolites occurred in the lung, the target organ for 3-methylindole, after administration of 3-methylindole. Nucleophilic substances which prevented alkylation of cellular macromolecules *in vitro* by the reactive metabolites of 3-methylindole decreased the pneumotoxic effect of 3-methylindole *in vivo*. The reactive metabolite of 3-methylindole appears to be detoxified by preferential, spontaneous and glutathione S-transferase catalyzed, conjugation with glutathione. In favour of this conclusion, glutathione protected cellular macromolecules, *in vitro*, from alkylation by the reactive metabolite of 3-methylindole and glutathione S-transferases increased the protective effect of glutathione. Depletion of glutathione increased the pneumotoxic effect of 3-methylindole *in vivo*. The pneumotoxic effect of 3-methylindole can be decreased by:

1) Preventing the formation of the reactive metabolite:

Results of this present work have established that the covalent binding of 3-methylindole is catalyzed by a cytochrome P-450-dependent mixed function oxidase enzyme system. The use of inhibitors of mixed function oxidases would be prohibited by the fact that these compounds would inhibit detoxifying enzymes as well as toxifying ones, making the overall effect of these compounds on foreign compound-induced lung injury unpredictable (Boyd, 1976).

2) Preventing alkylation of cellular macromolecules by providing alternative nucleophilic sites for covalent binding with the electrophilic metabolite of 3-methylindole:

This has been achieved in this present study by the use of L-cysteine.

The pathogenesis of the 3-methylindole-induced lung lesions appears to commence with alkylation of cellular macromolecules with the reactive metabolite of 3-methylindole. This leads to cellular injury and necrosis. Cellular injury can stimulate the release of mediators of anaphylaxis and thus cause inflammatory changes in the lung and systemic changes similar to those associated with anaphylaxis. Therefore, although antagonists of mediators of anaphylaxis have been reported to alleviate the immediate anaphylactoid-like reaction following administration of 3-methylindole (Atkinson et al, 1977), these antagonists did not alter the severity of 3-methylindole-induced lung lesions (Hammond, Carlson, Breeze and Selman, 1979).

From the studies reported here using chopped lung preparations it would appear that 3-methylindole does not directly cause the release of mediators of anaphylaxis. The release of these mediators is not the primary event in the disease process but is rather a result of the cytotoxic effects of the alkylation metabolite of 3-methylindole. Also 3-methylindole does not cause contraction of the pulmonary vein. Therefore a hydrodynamic imbalance across the alveolocapillary membrane caused by pulmonary venoconstriction also cannot be considered as the initial step in the pathogenesis of the 3-methylindole-induced pulmonary injury, as has been suggested by other investigators (Eyre, 1975).

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