This is to certify that the

dissertation entitled

The Role of the Platelet and Platelet Mediators in the Pulmonary Hypertensive Response to Monocrotaline Pyrrole

presented by

Patricia Elaine Ganey

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Pharmacology and Toxicology

Robert A Roth
Major professor

Date 9/9/86

THE ROLE OF THE PLATELET AND PLATELET MEDIATORS IN THE PULMONARY HYPERTENSIVE RESPONSE TO MONOCROTALINE PYRROLE

By

Patricia Elaine Ganey

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

1986

ABSTRACT

The Role of the Platelet and Platelet Mediators in the Pulmonary Hypertensive Response to Monocrotaline Pyrrole

by

Patricia Elaine Ganey

Monocrotaline pyrrole (MCTP) is a metabolite of the plant toxin monocrotaline. Administration of MCTP to rats produces pulmonary vascular injury, pulmonary hypertension (PH), and right ventricular enlargement (RVE). The mechanism by which MCTP causes PH is unknown, however platelets have been implicated in the response. The possibility that the platelet was contributing to MCTP-induced PH by releasing vasoactive mediators, specifically 5-hydroxytryptamine (5HT) and thromboxane A_2 (TxA_2), two mediators which cause vasoconstriction in the lung, was examined.

To determine whether platelet depletion attenuated the development of PH, MCTP-treated rats were co-treated with an anti-platelet serum (PAS) to reduce the circulating platelet count to 10-25% of normal. Treatment with a single dose of MCTP on day 0 produced lung injury, PH, and RVE by day 14. Rats co-treated with PAS so that they were moderately thrombocytopenic from days 6-8 did not develop PH or RVE. Indices of lung injury were not affected by co-treatment with PAS.

To examine whether 5HT was involved in the response to MCTP, the effect of MCTP on platelet 5HT content and the effect of co-treatment with a 5HT receptor antagonist on MCTP-induced toxicity was determined. The platelet content of 5HT was not affected by MCTP treatment. A dose of the $5HT_2$

receptor antagonist ketanserin which inhibited the 5HT-induced shape change in platelets and the 5HT-induced vasoconstriction in isolated, perfused lungs, did not attenuate the lung injury or RVE caused by MCTP. These results suggest that 5HT is not the sole contributor to PH due to MCTP.

To investigate a possible role for the arachidonic acid (AA) metabolite TxA_2 in MCTP-induced PH, release of TxA_2 was determined in isolated, perfused lungs and in platelets from treated rats. The effect of co-treatment with inhibitors of TxA_2 synthesis or activity on MCTP-induced PH was also examined. Treatment with MCTP was associated with an increased release of the TxA_2 metabolite, TxB_2, from isolated lungs perfused with buffer or blood. The increase in release was greater when lungs were perfused with blood, suggesting a blood element as a major source of TxB_2. Release of a stable metabolite of prostacyclin, an AA metabolite with activities that oppose those of TxA_2, from isolated lungs was not affected by treatment with MCTP in vivo. Generation of TxB_2 in platelet-rich plasma in response to aggregation by AA was not different for rats treated with MCTP and controls. Co-treatment with either a cyclooxygenase inhibitor (ibuprofen), a thromboxane synthetase inhibitor (Dazmegrel), or a thromboxane receptor antagonist (L-640,035) did not attenuate the development of lung injury, PH, or RVE. Thus, TxA_2 does not appear to be the sole contributor to MCTP-induced PH.

These results indicate that modest depletion of platelets prevents MCTP-induced PH and RVE. Neither of the two platelet-derived mediators examined, 5HT or TxA_2, appear to be necessary for the development of lung injury, PH, or RVE due to MCTP.

ACKNOWLEDGEMENTS

I would like to express my sincerest thanks to my major advisor and friend, Dr. Bob Roth, for ideas and encouragement, for insisting on meticulous research, for always believing in me, and for never letting me forget that all of this was fun. I thank the rest of my Guidance Committee, Drs. Theodore M. Brody, Gregory D. Fink, and James W. Aiken, for their time, attention, and helpful advice.

My thanks go to Drs. Ron Slocombe and James Reindel for numerous hours and endless patience in assistance with pathology studies, and to Dr. Tom Bell for his expert advice on platelets. I would also like to thank Dr. Scott Walsh for his assistance in setting up the RIA.

I feel fortunate to have spent this time among colleagues who have been supportive and encouraging. My thanks go to Katie Sprugel, Leon Bruner, Laurie Carpenter, Lonnie Dahm, and Jim Hewitt for making this experience more enjoyable. I would also like to thank Lynn Georgic, Duane Kreil, Jeff Fagan, and Jim Wagner for their expert technical assistance, careful attention to detail, and just as importantly, for their enthusiasm for the project. I would also like to express thanks to Kaelyn Boner and Nancy Shannon for their contributions to this project.

Special thanks go to Diane Hummel, not only for her excellent performance in typing this dissertation, but also for her help over the past four years.

Some of the drugs used for this research were supplied as gifts. I would like to thank Janssen Pharmaceutica (Beerse, Belgium) for the gift of ketanserin, Mr.

Peter Chelune of The Upjohn Company (Kalamazoo, MI) for supplying ibuprofen and U46619, Pfizer Central Research (Sandwich, Kent, England) for the gift of Dazmegrel, and Dr. Ford-Hutchinson of Merck-Frosst (Canada) for supplying L-640,035.

I would also like to acknowledge and thank those who provided financial support for my graduate work. My stipend and travel allowance came from NRSA Training Grant GM07392 and NIH Training Grant HL07404, and in part from the Hazleton Laboratories Graduate Fellowship Award administered by the Society of Toxicology. My research was supported by US Public Health Service grant ES02581.

Finally, my deepest appreciation goes to all of my family, especially to my Mom and Dad, and to Robert, for their support and encouragement, and for their expression of pride in my achievements. And, to Rick, thanks for smiling through it all, and for constantly reminding me of all of the other important things in life.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

LIST OF ABBREVIATIONS

AA	arachidonic acid
ACE	angiotensin converting enzyme
AII	angiotensin II
BUN	blood urea nitrogen
CS	control, or pre-immune, serum
DMF	N,N-dimethylformamide
EC	endothelial cell
5HT	5-hydroxytryptamine
IBN	ibuprofen
KET	ketanserin
LDH	lactate dehydrogenase
LT	leukotriene
MCT	monocrotaline
MCTP	monocrotaline pyrrole
PAF	platelet-activating factor
PAP	pulmonary arterial pressure
PAS	anti-platelet serum
PDGF	platelet-derived growth factor
PG	prostaglandin
PGI_2	prostacyclin
PPP	platelet-poor plasma
PRP	platelet-rich plasma
PZA	pyrrolizidine alkaloid
RIA	radioimmunoassay
RVE	right ventricular enlargement
SGOT	serum glutamic oxalacetic transaminase
Tx	thromboxane

INTRODUCTION

I. Pyrrolizidine Alkaloids

A. General

Monocrotaline (MCT) is a member of a class of compounds referred to as the pyrrolizidine alkaloids (PZAs). The common chemical structure for this class of compounds is the pyrrolizidine ring, consisting of two fused 5-membered rings with a nitrogen atom at the center (Figure 1) (McLean, 1970). Many of the PZAs are toxic, and an unsaturated pyrrolizidine ring structure is an essential requirement for toxicity (Huxtable, 1979). To date, more than 150 PZAs have been identified and isolated, and the structures have been determined (Huxtable, 1979).

The plants that contain PZAs comprise 8 families and numerous genera that are widely distributed geographically (Bull et al., 1968; Huxtable, 1979). Some of the more common PZA-containing genera include Crotalaria (from which MCT is extracted), Senecio, and Heliotropium. Toxicity due to exposure to some PZAs is responsible for significant loss of livestock in the United States (Snyder, 1972) and other areas of the world, and for loss of human life as well. The occurrence and toxicity of PZAs has been reviewed (Bull et al., 1968; McLean, 1970; Huxtable, 1979).

B. Toxicity to Humans

Because symptoms of toxicity are often delayed following exposure and because detection of low concentrations of PZAs or their metabolites is

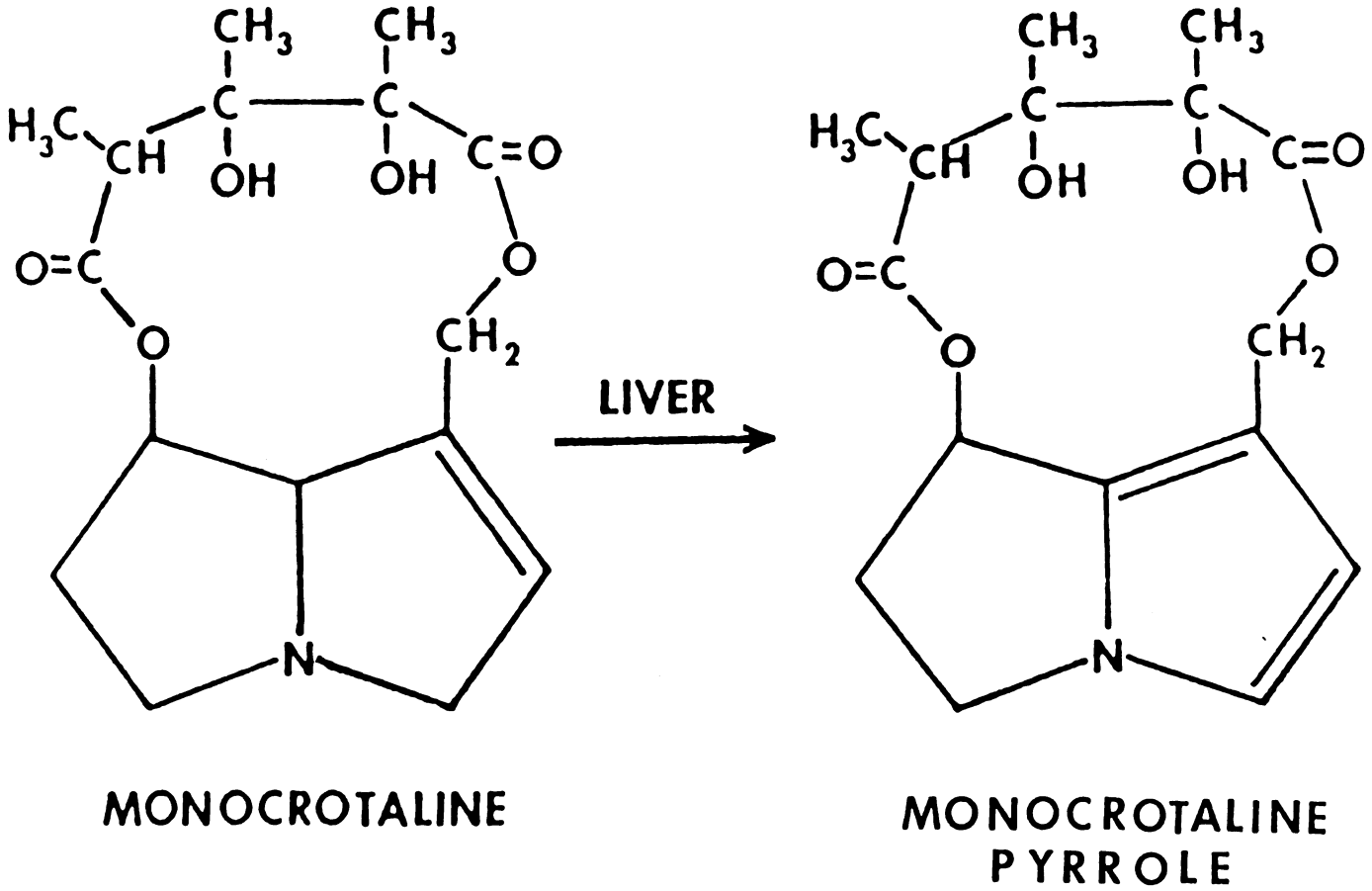

Figure 1. Structures of MCT and MCTP.

difficult, diagnosis of PZA poisoning is based on circumstantial evidence. Still, it is well established that humans exposed to PZAs develop veno-occlusive disease of the liver. Symptoms of this hepatic vascular disease include massive centrilobular congestion and necrosis, collagenous occlusion of small branches of the hepatic venous tree, development of collateral venous channels over the abdomen, and dilated sinusoids (Hill et al., 1951). Many PZA-intoxicated people die.

Exposure can occur by a number of mechanisms. Veno-occlusive disease, endemic in Jamaica, is thought to arise from the use of "bush" teas prepared from plants, some of which contain PZAs (Hill et al., 1951; Bras et al., 1954; Stuart and Bras, 1957). Grains have become contaminated with PZAs when PZA-containing plants grow as weeds in the same fields. Use of these grains to prepare bread results in low grade exposure and may be responsible for large outbreaks of liver disease such as was seen in 1975 in Afghanistan (Mohabbat et al., 1976) and India (Tandon et al., 1976). In the United States, use of the commercial preparation of gordoloba yerba caused PZA poisoning in small children in Arizona (Stillman et al., 1977; Fox et al., 1978). In addition, contamination of honey used for human consumption (Dienzer et al., 1977) or of the milk of cows which graze on the plants (Dickinson et al., 1976) can result in human exposure to PZAs.

C. Toxicity to Animals

PZA poisoning in animals has been recognized for quite some time. As long ago as 1787 British farmers suspected PZA-containing plants were harmful to livestock (Bull et al., 1968). The PZAs are toxic to a wide variety of species, and the symptoms associated with PZA toxicity have been given many different descriptive names. Chronic liver disease commonly occurs in pigs, dogs, and cattle, although the gastrointestinal system of cattle is also affected. Horses

develop mainly a neurological syndrome, sheep exhibit hemolytic disease (Huxtable, 1979), and chickens and turkeys develop lesions of the liver and lungs (Allen et al., 1960, 1963). PZAs are also carcinogenic and mutagenic in animals (Cook et al., 1950; Schoental et al., 1954). PZA poisoning represents a great economic loss, costing millions of dollars annually in the United States alone (Snyder, 1972).

As with humans, the diagnosis of PZA poisoning in grazing animals is largely based on circumstantial evidence, but the toxicity can be reproduced in laboratory animals given PZAs (Huxtable, 1979). The PZA which has attracted the most attention and which has been most extensively studied is MCT, and the remainder of this dissertation will be confined to a discussion of MCT.

II. Monocrotaline

A. General

The PZA monocrotaline (MCT) is found in the seeds and leaves of plants of the genus Crotalaria (Heath, 1969), and in the United States, largely in the seeds of Crotalaria spectabilis (IARC). C. spectabilis grows in other areas of the world, but was introduced in the United States in 1921 by the Florida Agricultural Experimental Station as a leguminous cover crop. C. spectabilis now grows wild in many southern states (Kay and Heath, 1969).

MCT is the monocrotalic acid ester of retronecine (Adams and Rodgers, 1939). Its structure, illustrated in Figure 1, has been identified using nuclear magnetic resonance and infrared and mass spectrometry (Culvenor and DalBon, 1964; Bull et al., 1968). It is a colorless, crystalline powder with a melting point of 202-203°C.

B. Pathophysiology

Numerous studies have been conducted examining the pathophysiology of PZAs or MCT. Various methods, as well as routes, of administration have been

employed, making comparison of these studies difficult. For example, many investigators have ground the seeds of C. spectabilis and have fed these to animals in the diet. Others have dissolved MCT in the drinking water. Both of these methods of chronic administration produce similar effects, but there may be subtle differences in the time course of those effects. Also, results from studies using these methods, in which the actual dose of MCT each animal received is merely estimated, are difficult to compare with results obtained from studies in which animals received a single administration of a known amount of MCT. The discussion below will concentrate on those studies in which MCT was administered, although studies using C. spectabilis seeds will be discussed when they add new information or clarify an issue. The effects of MCT on organs other than the lung will be briefly described, while pulmonary effects will be described in detail.

1. Species affected

MCT produces toxic effects in a wide variety of experimental animals including rats (Roth, 1981; Molteni et al., 1985), rabbits (Gardiner et al., 1965), mice (Miranda et al., 1981), dogs (Miller et al., 1981), and non-human primates (Allen et al., 1965; Raczniak et al., 1978). Guinea pigs (Chesney and Allen, 1973b), gerbils, and hamsters (Cheeke and Pierson-Goeger, 1983) are not susceptible to MCT toxicity. MCT intoxication has been reported in other species such as cattle (Becker et al., 1935), horses (Rose et al., 1957), hogs (Emmel et al., 1935), poultry (Thomas, 1934; Allen et al., 1960, 1963; Simpson et al., 1963), and goats (Dickinson, 1980). Human intoxication by MCT has also been reported (Kasturi et al., 1979).

2. Pharmacokinetics

Detailed pharmacokinetic analysis has not been performed for MCT because radiolabeled MCT with high specific activity is not available. Hayashi (1966) reported that 50-70% of ^{3}H-MCT is recovered unchanged in the

urine within 3 hours, and 30% is recovered in the bile as a metabolite. Pyrrole derivatives of MCT (Figure 1), assayed as Ehrlich-positive materials, are detected in tissues within minutes after administration of MCT (Mattocks and White, 1970), reach a peak at about 90 minutes, and then decrease to low concentrations by 48 hr (Allen et al., 1972; Mattocks, 1972).

3. Hepatotoxicity

Veno-occlusive disease of the liver, described above, has been the most frequently observed effect of PZA poisoning. Animals treated with MCT develop centrilobular focal cell necrosis, and portal hepatic veins become dilated and congested (Schoental and Head, 1955; Turner and Lalich, 1965; Merkow and Kleinerman, 1966). Enlarged parenchymal cells have also been observed (Turner and Lalich, 1965). Prothrombin time increases (Rose et al., 1945) and plasma glutamic pyruvic transaminase activity increases (Roth et al., 1981). Biliary excretion of indocyanine green is depressed although bile production is not altered (Roth et al., 1981).

4. Renal toxicity

The majority of renal changes caused by MCT occur in the glomeruli, glomerular capillaries, and afferent arterioles (Hayashi and Lalich, 1967). Glomeruli become swollen, and thrombosis of glomerular capillaries and afferent arterioles has been reported. Interlobular arteries thicken and the media hypertrophies. In rats fed ground C. spectabilis seeds for up to 8 months (Masugi et al., 1965; Carstens and Allen, 1970), the most severe case of intoxication caused replacement of 75% of the glomeruli with a homogenous periodic acid Schiff-positive material, and glomerular capillaries were only occasionally discernible (Carstens and Allen, 1970).

Alterations in renal function have been reported in animals which received MCT in the drinking water for 4 weeks (Roth et al., 1981).

Consistent with the histopathologic picture drawn from the above studies, blood urea nitrogen (BUN) increased, indicating impaired glomerular filtration. Necrosis of renal tubules has been reported (Ratnoff and Mirick, 1949), and the accumulation of organic ions, an index of proximal tubular secretory functions, was altered by MCT treatment (Roth et al., 1981).

Thus, the toxic effects of MCT on the kidney include vascular damage, especially at the level of the glomerular capillaries and afferent arterioles, and impaired glomerular and tubular function.

5. Cardiac effects

Chronic administration of C. spectabilis seeds in the diet (Kay et al., 1967b; Hislop and Reid, 1974; Meyrick et al., 1980) or MCT in the drinking water (Roth et al., 1981; Molteni et al., 1985) produces right ventricular enlargement (RVE). This effect is also produced by a single administration of relatively low doses of MCT (Kameji et al., 1980; Ghodsi and Will, 1981; Hilliker et al., 1982; Gillespie et al., 1985). RVE follows the development of pulmonary hypertension and is believed to be a compensatory response to the increased pulmonary vascular resistance. It has also been reported that when animals previously fed C. spectabilis seeds are allowed to eat unadulterated feed, they recover from pulmonary hypertension, and RVE is no longer evident (Hislop and Reid, 1974).

The heart has not received much attention in histopathological studies of MCT toxicity. One detailed investigation demonstrated no alterations in the left ventricle, but the number and size of mitochondria in the right ventricular myocardium increased 14 days after treatment with MCT (Kajihara, 1970). Irregularities of the right ventricle progressed such that by 25 days after treatment a majority of the right ventricular muscle cells were hypertrophied and contained enlarged, bizarre-shaped nuclei. Changes in myofibrils suggested that

the heart was responding to a pressure overload. Golgi vesicles were electron-dense, cisternae of the endoplasmic reticulum were enlarged, and there were a larger number of free ribosomes in the cytoplasm, indicative of increased RNA synthesis. Others have demonstrated that protein and collagen content increase in the right but not left ventricles of MCT-treated rats (Lafranconi et al., 1984), and that protein synthesis increases in parallel with right ventricular weight (Huxtable et al., 1977). The DNA/RNA ratio decreased in the right ventricles of MCT-treated rats, largely due to an increase in RNA content (Lafranconi et al., 1984). Although an increase in RNA would suggest a hypertrophic response, DNA synthesis was not measured in this study so that a hyperplastic response could not be ruled out.

All of these changes suggested that the right ventricle was undergoing increased energy production to compensate for the excess work it had to perform. However, most myofilaments were normally arranged, and the mean diameter of thick filaments was not different from that of controls, as might have been the case if these rats were experiencing heart failure (Kajihara, 1970). Werchan and coworkers (1986) agree that right ventricular function is not impaired in MCT-treated rats. Right ventricular systolic pressure and the maximum rise and fall in pressure development (+dp/dt), measured using an isolated Langendorff preparation, were elevated at several preloads in hearts from MCT-treated rats relative to controls.

In summary, RVE develops in response to MCT-induced pulmonary hypertension. There appears to be a hypertrophic element to the enlargement although protein and collagen content increase as well. Right ventricular function does not seem to be impaired by MCT treatment.

6. Pulmonary toxicity

The pathophysiology of MCT intoxication is progressive. Whether administered chronically or as a single injection, lesions and alterations observed worsen with time. An effort will be made to address this issue in the discussion of pulmonary pathology. It should also be mentioned that lesions observed microscopically are generally multifocal in nature rather than diffuse, and the severity of lesions varies within a lung (Turner and Lalich, 1965; Merkow and Kleinerman, 1966; Valdivia et al., 1967).

a. Gross changes

Following treatment with doses of MCT which produce pulmonary toxicity, rats become dyspnic. Their fur takes on a ruffled appearance and they become listless. Food consumption as well as the rate of body growth decreases. Severe respiratory distress and cyanosis after about 2 or more weeks from the onset of treatment generally indicate impending death of the animal. Grossly, regions of the lung are dark brown and atelectic, and the lungs are heavy and fluid-filled (Schoental and Head, 1955; Turner and Lalich, 1965; Merkow and Kleinerman, 1966; Gillis et al., 1981).

b. Microscopic changes

1) Changes in endothelial cells. The earliest changes noted in the lung following treament of rats with MCT are in endothelial cells (ECs), suggesting that this cell is a target of MCT toxicity. In one report, cytoplasmic alterations were seen within 24 hours after a single injection and progressed throughout the course of the disease (Valdivia et al., 1967). At the capillary level, alterations include alternate thinning and thickening of EC cytoplasm (Valdivia et al., 1967).

Later in the development of toxicity, swollen ECs, occasionally occluding the vessel lumen, are seen in capillaries (Turner and Lalich,

1965; Valdivia et al., 1967; Molteni et al., 1984), pulmonary arterioles (Turner and Lalich, 1965), and small muscular pulmonary arteries (Turner and Lalich, 1965; Merkow and Kleinerman, 1966). ECs of pulmonary veins are not affected (Turner and Lalich, 1965). Associated with swollen ECs are acellular (Merkow and Kleinerman, 1966) or fibrin thrombi (Turner and Lalich, 1966). Injured ECs have prominent, enlarged nuclei and an increased number of cytoplasmic organelles. Similar changes in ECs are seen in MCT-treated monkeys (Chesney and Allen, 1973a) and rats fed C. spectabilis seeds (Kay et al., 1969; Hislop and Reid, 1974).

In rats fed C. spectabilis seeds, Meyrick and Reid (1982) demonstrated a proliferation of ECs in the alveolar wall and in intra-acinar arteries and veins. ^{3}H-Thymidine uptake peaked at 7 and again at 21 days after the animals had started receiving the seeds. This suggested an early and late hyperplastic response of ECs. Unfortunately, the investigators did not look earlier than day 7, which would have been of interest in light of the report of EC changes within one day after treatment with MCT (Valdivia et al., 1967). In fact, given that the EC is thought to be a target of MCT toxicity, the study of very early changes in ECs has been neglected, and further investigation and more detailed description is warranted.

2) Changes in pulmonary vessels. The vascular changes that occur following MCT treatment, including the EC changes described above, are numerous and progressive. The most consistent finding in MCT-treated rats is an increased thickness of the medial layer of pulmonary vessels (Turner and Lalich, 1965; Hayashi and Lalich, 1967; Ghodsi and Will, 1981; Kay et al., 1982a; Hayashi et al., 1984; Molteni et al., 1984). This is observed as soon as 8 days after MCT treatment, and appears earlier and to a greater degree in smaller pulmonary blood vessels (Turner and Lalich, 1965; Hayashi and Lalich, 1967). The increased medial size has been attributed to a proliferation of circularly-oriented smooth

muscle within the internal and external elastic laminae (Ghodsi and Will, 1981), although smooth muscle has also been observed outside the elastic laminae (Turner and Lalich, 1965). Increased numbers of elastic laminae have been reported as well (Molteni et al., 1984).

Smooth muscle extends to smaller, normally nonmuscular vessels as well (Langleben and Reid, 1985). This occurs by day 21 following a single injection of MCT, although earlier times have not been investigated. In rats fed C. spectabilis seeds, this extension of vascular smooth muscle is observed by day 7 in pulmonary vessels at the level of respiratory bronchioles, and later in pulmonary vessels at the level of smaller airways (Meyrick and Reid, 1979).

Meyrick and Reid (1982) reported an increased ^{3}H-thymidine uptake in smooth muscle cells of pulmonary arteries of C. spectabilis-fed rats within 3 days. However, the increase was extremely small and occurred in only one of three treated rats. In MCT-treated rats, smooth muscle cells have an increased number of mitochondria, increased rough endoplasmic reticulum, and prominent Golgi, suggesting a hypertrophic response. An electron-dense, amorphous material that is not collagen has been described surrounding the elastic laminae and existing between and around the smooth muscle cells extending into the adventitia (Merkow and Kleinerman, 1966; Heath and Smith, 1978). Some other adventitial changes have been noted, including increased collagen and proliferation of fibroblasts, but only in rats receiving MCT chronically (Merkow and Kleinerman, 1966).

A diffuse, necrotizing vasculitis has been described by some investigators (Merkow and Kleinerman, 1966) but not others (Hayashi and Lalich, 1967; Kay et al., 1982a). This was characterized by a periodic acid Schiff-positive material within the walls and lumina, was confined to small pulmonary arteries and arterioles, and was associated with focal hemorrhage, edema and

congestion. No leukocyte infiltration or inflammation was noted in smaller vessels, although in larger vessels the pseudopodia of polymorphonuclear cells (PMNs) and macrophages were seen to extend through fenestrations of the internal elastic laminae (Merkow and Kleinerman, 1966). Capillary thrombosis, associated with edema, hemorrhage, and necrosis, was evident before arterial thrombosis was observed (Hayashi and Lalich, 1967). Thrombi have been reported to contain fibrin, platelets, and red blood cells (Merkow and Kleinerman, 1966; Turner and Lalich, 1967; Heath and Smith, 1978; Kay et al., 1982a; Hayashi et al., 1984).

Changes in pulmonary vessels of MCT-treated rats are consistent with those seen in rats fed C. spectabilis seeds (Masugi et al., 1965; Kay and Heath, 1966; Kay et al., 1967b; Hislop and Reid, 1974; Meyrick and Reid, 1979), although a few differences bear mentioning. No changes have been reported for the pulmonary veins of MCT-treated rats, but smooth muscle cells in the pulmonary veins of rats fed seeds were swollen and were evaginating toward the EC (Heath and Smith, 1978). Another difference is that a decrease in the number of peripheral arteries has been observed qualitatively (a decrease in background haze in contrast radiography) and quantitatively (a decrease in the number of arteries relative to the number of alveoli) (Hislop and Reid, 1974; Meyrick and Reid, 1979) in rats fed C. spectabilis seeds, whereas there was no decrease in the total number of small pulmonary blood vessels in MCT-treated rats (Kay et al., 1982b). Ghost arteries, thought to be remnants of obliterated vessels, have also been observed in rats fed seeds (Hislop and Reid, 1974). It has been suggested that certain fixation procedures, such as the vascular perfusion technique employed by Meyrick and Reid (1979), artifactually decrease the number of pulmonary blood vessels observed histologically (Mooi and Wagenvoort, 1983), so that this difference between MCT treatment and C. spectabilis feeding

may be explained on the basis of fixation procedures. Or, these differences may be due to the difference in chronic administration of seeds in the diet and acute administration of MCT by injection. Alternatively, the differences may be due to other substances (e.g., perhaps other PZAs) in the seeds, or to variations in the development of toxicity by these two methods of exposure.

3) Parenchymal changes. In one report, interstitial alveolar edema was observed as early as 4 hours after treatment with a single injection of MCT (Valdivia et al., 1967). This progressed such that by one week after treatment perivascular edema was noted around small pulmonary vessels (Valdivia et al., 1967; Molteni et al., 1984). Hyaline membranes, mainly fibrin and cellular debris, were associated with areas of severe alveolar edema.

Simultaneous with the early alveolar edema, interstitial cells of the alveolar wall become swollen. Between 2 days and 2 weeks these cells migrate into the alveolar spaces (Valdivia et al., 1967; Kay et al., 1982a). Focal swelling of elastic membranes has been reported within 24 hours, and by 2 weeks elastic tissue is decreased and collagen bundles appear in the alveolar walls (Valdivia et al., 1967). Hemorrhage and fibrosis are late changes (Turner and Lalich, 1965; Merkow and Kleinerman, 1966; Kay et al., 1969).

Proliferation of epithelial cells has also been observed. Type II pneumocytes become enlarged, with increased numbers of cytoplasmic organelles (Masugi et al., 1965; Valdivia et al., 1967; Kay et al., 1982a). In C. spectabilis-fed rats, bronchiolar epithelium extends to alveolar ducts and alveolar spaces, replacing the normal alveolar epithelium (Kay and Heath, 1966).

Peribronchiolar lymphatics become dilated and contain protein and red blood cells (Hayashi and Lalich, 1967; Kay et al., 1969). As early as 4 hours after treatment, lymphocytes and mast cells are observed in the

swollen alveolar walls (Takeoka et al., 1962; Hayashi and Lalich, 1967; Valdivia et al., 1967; Kay et al., 1967a, 1969; Sugita et al., 1983b). Abnormal alveolar macrophages are observed, large and foamy in appearance and containing numerous electron-dense granules (Kay and Heath, 1966; Sugita et al., 1983b). The number of abnormal macrophages increases with increasing doses of MCT (Sugita et al., 1983b).

4) Summary. MCT causes a progressive disease in which the number and degree of changes increases with time after treatment. The EC appears to be a primary target in MCT toxicity, and is affected early in treatment. The ECs of smaller vessels are most severely affected. Changes in vascular smooth muscle lag behind alterations in ECs, generally taking more than a week to develop. Some smooth muscle extension is observed, but medial thickening is the most prominent alteration. Like the EC changes, the latter effect is observed earliest and to the greatest degree in smaller vessels. Alterations in the airway include epithelial proliferation and infiltration of mast cells, lymphocytes and macrophages. How these changes relate to each other, or to the overall response to MCT, is not well understood.

c. Hemodynamic changes

Pulmonary arterial pressure (PAP) increases in animals which are fed C. spectabilis seeds (Kay et al., 1967b; Meyrick et al., 1980; McNabb and Baldwin, 1984) or which receive a single injection of MCT (Chesney and Allen, 1973a; Ghodsi and Will, 1981; Olson et al., 1984a). The increase in PAP is protracted, and is associated with the eventual development of RVE.

Pulmonary hypertension is a secondary event to EC injury. It is unclear whether the elevation in PAP can be attributed solely to the observed increase in vascular smooth muscle or whether there is a vasoconstrictive component as well. In chronic human primary pulmonary hypertension it is

believed that vasoconstriction precedes and is the cause of vascular remodelling (Voelkel and Reeves, 1979). However, eight days after administration of MCT, significant medial thickening of small pulmonary vessels was reported while only a small tendency toward an increase in PAP was observed (Ghodsi and Will, 1981). This is consistent with the finding of Kay and coworkers (1982a) that medial thickening preceded increases in right ventricular systolic pressure (PRVS). However, in this latter study medial thickening was first elevated on day 7, and although the increase in PRVS in MCT-treated rats did not reach statistical significance until day 10, the magnitude of the increase in MCT-treated rats was the same on days 7 and 10. Therefore, this particular study did not resolve whether increases in pulmonary vascular pressure coincide with or follow vascular remodelling. The results of these two studies might suggest that some vascular remodelling precedes the development of pulmonary hypertension, but do not rule out the possibility that vasoconstriction contributes to increases in PAP. In support of the latter hypothesis, Watanabe and Ogata (1976) observed increases in right ventricular pressure prior to medial thickening of vessels in MCT-treated rats.

Meyrick and coworkers (1980) suggested that vasoconstriction was not involved in the early development of increased PAP in C. spectabilis-fed rats. This conclusion was based on the assumption that, if vasoconstriction were involved, cardiac index would be depressed and pulmonary vascular resistance would be elevated. When these investigators first detected pulmonary hypertension at day 14, cardiac index was elevated in treated rats and pulmonary vascular resistance was not different from control rats. There may be a problem with this conclusion that vasoconstriction does not contribute to PAP based on a comparison of cardiac index and pulmonary vascular resistance, however, because by day 14 body weight was significantly reduced in treated rats. It is not certain

whether reduced body weight in MCT-treated rats is due to loss of body fat or to failure to grow, but this may be significant when comparing cardiac index (which is normalized to body weight) and pulmonary vascular resistance (which is normalized to cardiac index) in MCT-treated and control rats. For example, if reduction of body weight in treated rats is due to a loss of body fat, cardiac output might not be expected to decrease commensurately with body weight. The result would be an elevation in cardiac index in treated rats relative to control rats. In the same manner, pulmonary vascular resistance would be underestimated in treated rats. Therefore, to conclude that vasoconstriction is not contributing to increased PAP on the basis of these results may be a misinterpretation.

Later in the development of pulmonary hypertension (21 days) due to C. spectabilis seeds, pulmonary vascular resistance was elevated and cardiac index was depressed (Meyrick et al., 1980; McNabb and Baldwin, 1984). This suggests that vasoconstriction may play a role later in the development of pulmonary hypertension.

By 21 days after the start of seed feeding, arterial and venous oxygen contents were depressed, suggesting impaired gas exchange (Meyrick et al., 1980; McNabb and Baldwin, 1984). Blood pH and hematocrit were not altered by feeding C. spectabilis seeds. Platelet number was affected by MCT treatment: the number of circulating blood platelets was decreased during days 2-10 following a single injection of MCT, then was increased above baseline by day 14 (Hilliker et al., 1982).

In summary, although EC injury has been reported within one day of treatment with MCT, PAP does not increase until day 7 or later. The relationship between changes in ECs and vascular remodelling or pulmonary hypertension, or between vascular remodelling and increases in PAP have not been completely delineated. More detailed studies of alterations between day 1 and

day 7 may improve our understanding of MCT pneumotoxicity. Examination at intervals more frequent than one week may also enhance our knowledge of mechanisms of MCT's toxicity.

d. Changes in vascular smooth muscle responsiveness

Alterations in responsiveness to vasoactive agents have been observed in isolated vessel segments and isolated, perfused lungs from MCT-treated rats. Vasoconstriction in response to angiotensin II, norepinephrine, or KCl was elevated in segments of pulmonary arteries isolated from rats treated 4 days earlier with a single injection of MCT compared to control rats (Altiere et al., 1986b). By 7 days post-treatment, the response had returned to control, and by day 14 after administration of MCT, the response in vessels from treated rats was depressed compared to controls. Response to vasodilation induced by isoproterenol or acetylcholine was not altered at day 4 or 7, but was reduced in vessels isolated from MCT-treated rats at day 14 (Altiere et al., 1986b). By 14 (Altiere et al., 1986b) or 21 (Coflesky et al., 1985) days after a single injection of MCT, vessels from treated rats demonstrated greater compliance and a reduced ability to generate force. This was associated with increased medial and adventitial areas and decreased lumen size (Coflesky et al., 1985). At this time the resting membrane potential of small pulmonary arteries was hyperpolarized and the resting membrane potential of main pulmonary arteries was depolarized (Suzuki and Twarog, 1982).

Altered responsiveness has also been observed in isolated lungs of MCT-treated rats (Gillespie et al., 1986). Similar to obervations in isolated pulmonary arterial segments (Altiere et al., 1986b), the vasoconstrictive response to angiotensin II in isolated, perfused lungs was elevated in MCT-treated rats at days 4 and 7, but not at day 14. Vasoconstriction induced by alveolar hypoxia was also greater in lungs of treated rats at day 4, but was not different

from control at day 7 or 14. The response to KCl in isolated, perfused lungs was not altered by treatment with MCT.

In summary, these results indicate that in preparations of isolated pulmonary arteries and isolated lungs, the pulmonary vasculature of MCT-treated rats is increased in responsiveness to certain vasoactive agents shortly after treatment, and hypo-responsive at later times. This might suggest that early in the development of MCT-induced pulmonary hypertension enhanced vasoconstriction to mediators present in the pulmonary circulation could cause elevations of PAP. This might also suggest that later in the development of pulmonary hypertension, when vascular remodelling and increased vascular smooth muscle have been reported, excessive vasoconstriction is not required to maintain elevated PAP.

e. Changes in endothelial cell function

In addition to providing a semipermeable barrier separating blood from parenchyma, the pulmonary endothelium serves a variety of metabolic functions. The luminal surface of these cells is endowed with transport structures, enzymes, and receptors accessible to blood constituents, and these permit the ECs to perform biosynthetic and metabolic activities. EC injury due to MCT is associated with alterations in some of these functions, which will be described here briefly and in more detail in a later section.

Angiotensin-converting enzyme (ACE), located in calveolae on the luminal side of pulmonary ECs (Ryan and Ryan, 1984), converts angiotensin I to angiotensin II (AII) and inactivates bradykinin. Molteni and coworkers (1984) reported that, when rats were given MCT in the drinking water, ACE activity in lung homogenates was increased after one week, but returned to normal and then was decreased by 6-12 weeks. Decreased ACE activity was observed whether the activity was expressed on the basis of the whole lung, wet weight, or protein

content (Molteni et al., 1985). Using a similar MCT treatment regimen, Lafranconi and Huxtable (1983) observed a decrease in lung ACE activity when normalized to protein content, however, since MCT treatment elevated protein concentration, whole lung ACE activity was not decreased. After a single injection of MCT, lung ACE activity was decreased by 7-10 days whether expressed on the basis of wet weight, protein content (Keane et al., 1982; Hayashi et al., 1984) or whole lung (Keane and Kay, 1984). Serum ACE was not altered by MCT treatment.

Removal of biogenic amines, such as 5-hydroxytryptamine (5HT) and norepinephrine (NE), is another function of pulmonary endothelium. This involves carrier-mediated uptake into the ECs and subsequent inactivation by monoamine oxidases (MAO) (Roth, 1985). Treatment with MCT in the drinking water is associated with impaired removal of perfused 5HT in isolated lungs by day 14 and impaired metabolism by day 21 (Gillis et al., 1978; Huxtable et al., 1978; Roth et al., 1981), although MAO activity is not altered (Gillis et al., 1978). 5HT removal is impaired as early as 5 days after a single injection of MCT (Hilliker et al., 1982). It has been reported that NE removal is also depressed in isolated lungs from MCT-treated rats (Gillis et al., 1978; Hilliker et al., 1984b), although Huxtable and coworkers (1978) reported that NE removal is not altered. This discrepancy may be due to the fact that Huxtable and coworkers (1978) perfused lungs at room temperature while other reports have been from isolated lungs perfused at physiological temperature.

f. Biochemical changes

1) Lavage fluid protein concentration and lactate dehydrogenase activity. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme, and its release into the airways is a nonspecific, yet sensitive indicator of cell injury. Elevation of protein concentration in the cell-free lavage fluid recovered

from airways is also an index of cell injury. Protein concentration and LDH activity are increased in the lavage fluid of rats treated with MCT (Roth, 1981; Roth et al., 1981). Serum LDH activity and protein are not altered.

2) Polyamines. The polyamines (spermidine and spermine) and their precursor putrescine are believed to have an essential role in cellular growth and proliferation (Heby, 1981). Treatment with MCT is associated with an early increase in the lung of activities of essential enzymes in the polyamine biosynthetic pathway (Olson et al., 1984a,b). Polyamine concentrations are also increased in the lungs of rats one week after MCT treatment.

g. Changes in respiratory mechanics

Although MCT is recognized as a pulmonary toxicant, alterations in respiratory mechanics due to MCT have been largely ignored as an area of investigation. However, Gillespie and coworkers (1985) demonstrated recently that a single injection of MCT reduces total lung capacity, tidal volume, respiratory frequency, and dynamic or quasi-static compliance by day 20. Lung resistance was elevated in MCT-treated rats, and the coefficient of diffusion, an index of gas exchange, was reduced. These results are consistent with the parenchymal changes described, and indicate that, at least late in the development of pneumotoxicity, MCT-treated rats are in respiratory distress and have a reduced capacity for gas exchange. This is consistent with the report that arterial and venous oxygen contents are reduced by day 21 after the introduction of C. spectabilis seeds into the diet of rats (Meyrick et al., 1980).

C. Metabolism and Bioactivation

1. General

Although little is known about the mechanism by which MCT causes toxicity, it is generally accepted that injury is not due to MCT itself, which is relatively stable and non-toxic; rather, MCT (as well as other PZAs)

requires bioactivation. This is mediated in the liver by cytochrome P450-containing mixed function oxidase enzymes. This contention is supported by a number of observations. Microsomes prepared from liver, but not lung, metabolize PZAs to N-oxide and pyrrole derivatives, a reaction which is dependent on the presence of reduced NADP and oxygen (Mattocks and White, 1971). The conversion is inhibited by carbon monoxide or SKF-525A, and is increased in liver microsomes of phenobarbital-treated rats (Mattocks and White, 1971; White and Mattocks, 1971; Chesney et al., 1974b). Liver slices produce pyrroles when exposed to MCT or PZAs in vitro, however lung slices do not (Mattocks, 1968; Hilliker et al., 1983c). Pretreatment with inducers or inhibitors of cytochrome P450 increase or decrease, respectively, the severity of hepatic, renal, and pulmonary lesions and the lethality of MCT in vivo (Tuchweber et al., 1974). So it appears that one or more toxic metabolites are produced in the liver, and then leave the liver and travel via the circulation to other tissues to produce injury (Mattocks, 1968).

Some PZA metabolites, such as N-oxide derivatives, are relatively non-toxic (Lalich and Ehrhart, 1962; Mattocks, 1971; Culvenor et al., 1976). Many investigators believe that the toxic metabolites are pyrrole derivatives (Figure 1). Following administration of PZAs, pyrroles are covalently bound to injured tissues (Mattocks, 1968; Allen et al., 1972) and are excreted in the urine (Mattocks, 1968; Hsu et al., 1973). The amount of pyrroles produced in vitro or in vivo correlates with the relative toxicity of PZAs (Mattocks and White, 1971; Mattocks, 1972), and the amount of pyrrole found in tissue correlates with the degree of tissue injury (Mattocks, 1972). Pretreatment with phenobarbital increased the amount of pyrrole found in liver homogenates and increased the severity of liver and lung lesions and the lethality of MCT, whereas pretreatment with chloramphenicol had the opposite effect (Allen et al., 1972). In addition,

perfusion of isolated livers with MCT caused the appearance of pyrroles in the effluent, and when isolated lungs were perfused with this effluent, pulmonary injury was observed (Lafranconi and Huxtable, 1984).

In summary, MCT is bioactivated in the liver to one or more pyrrole metabolites which then pass to the lung and covalently bind to macromolecules. It is not known to which macromolecules pyrroles bind, however it is likely that this binding is involved in producing the injury that ultimately develops.

2. Monocrotaline pyrrole

a. Similarities in pathophysiologic effects of MCT and MCTP

One pyrrole derivative of MCT is monocrotaline pyrrole (MCTP) (Figure 1). When administered to animals, MCTP reacts with the first capillary bed it encounters to produce injury. When injected subcutaneously, MCTP causes a transient necrosis at the site of injection, and ultimately EC damage (Hooson and Grasso, 1975). When MCTP is given in a mesenteric vein, liver injury ensues (Butler, 1970). Administration of low doses of MCTP in a tail vein results in pulmonary vascular injury very similar to that seen following treatment with MCT (Butler, 1970; Bruner et al., 1986).

Approximately two days after a single administration of a low dose (2-5 mg/kg) of MCTP, alveolar edema, congestion, and hemorrhage were observed (Butler et al., 1970). By 1 week, the alveolar wall was thickened, and fibrin and numerous macrophages were present (Butler, 1970; Butler et al., 1970; Chesney et al., 1974a; Raczniak et al., 1979). The nuclei of capillary ECs were enlarged and the capillary lumen was often occluded. The severity of these lesions increased with time, and vessel leak and abnormalities of epithelial cells were observed after 2-3 weeks (Butler, 1970; Plestina and Stoner, 1972).

Smooth muscle cell proliferation has been observed in the media of pulmonary arteries of rats treated with a low dose of MCTP, although the time course of this change has not been delineated (Chesney et al., 1974a; Lalich et al., 1977). Interstitial fibrosis, dilated lymphatics, and platelet-containing thrombi have also been reported (Butler et al., 1970; Chesney et al., 1974a; Lalich et al., 1977; Raczniak et al., 1979).

Increases in lavage fluid protein concentration and LDH activity were observed after treatment with low doses of MCTP (Bruner et al., 1983; Hilliker et al., 1984a). Cardiac index decreased simultaneous with an increase in pulmonary vascular resistance (Raczniak et al., 1979). Pulmonary hypertension developed (Chesney et al., 1974a; Bruner et al., 1983) and eventually led to RVE (Chesney et al., 1974a; Lalich et al., 1977; Bruner et al., 1983; Hilliker et al., 1984a). Removal of biogenic amines was also impaired in isolated lungs and lungs slices from MCTP-treated rats (Hilliker et al., 1983c, 1984a).

Higher doses (10-30 mg/kg) of MCTP accelerated the development of pulmonary injury, and most treated rats died within 48 hours (Butler et al., 1970; Plestina and Stoner, 1972; Hurley and Jago, 1975). Death was preceded by pleural effusion and accumulation of edema fluid in the perivascular, peribronchiolar, and interstitial tissue, as well as in the alveolar lumens (Plestina and Stoner, 1972; Hurley and Jago, 1975). This was accompanied by increased vascular leak, largely from capillaries in the alveolar wall, which was not evident until 15 hours after treatment (Plestina and Stoner, 1972; Hurley and Jago, 1975). Gaps were observed between EC, many of which were swollen and contained prominent nuclei, and platelet aggregates were commonly noted in vessel lumens (Plestina and Stoner, 1972; Hurley and Jago, 1975). Alveolar walls appeared thickened and Type I cells were hypertrophic (Hurley and Jago, 1975). Thus,

higher doses of MCTP produce vascular leak and pronounced fluid accumulation in the lungs which result in death of the animal within 48 hours.

When MCTP was injected into the pulmonary artery of isolated lungs most pyrrolic material was retained by the lungs within the first 30 seconds (Plestina and Stoner, 1972). When the lungs were stained with Ehrlich reagent to detect pyrrole moieties, staining was widely distributed through the lung tissue, but was most intense in the walls of larger blood vessels (Plestina and Stoner, 1972). Lung injury was also produced in isolated lungs by MCTP (Hilliker and Roth, 1985b). Wet lung weight, lavage fluid protein concentration, and perfusion pressure increased, and metabolism of perfused 5HT decreased after injection of a moderate dose of MCTP directly into the pulmonary artery. These results suggest that most of the MCTP administered probably reacts with the pulmonary vasculature on first pass through the pulmonary circulation, and that direct exposure to MCTP can produce pulmonary injury in isolated lungs.

In summary, as with MCT, treatment with MCTP causes early changes in EC. Changes in pulmonary epithelium and vascular remodelling similar to those seen with MCT also follow treatment with MCTP. And, as with MCT, there are deficiencies in our knowlege of the development of MCTP pneumotoxicity. Alterations in ECs and pulmonary vasculature shortly after treatment (i.e., before day 2) have not been examined after giving low doses that result in the eventual development of pulmonary hypertension. The progression of changes in the pulmonary vasculature has also not been detailed. Finally, how the lesions that occur at a cellular level correlate with, or are responsible for, biochemical and functional changes has not been satisfactorily addressed. Investigation in each of these areas could enhance our knowledge of the mechanism by which MCT and MCTP produce pneumotoxicity and pulmonary hypertension.

b. Differences in pathophysiologic effects of MCT and MCTP

Although the similarities in the pathophysiology of MCT and MCTP are numerous, a few (minor) differences exist. The decrease in circulating platelet number observed following treatment with MCT (Hilliker et al., 1982) was not seen with MCTP treatment: platelet numbers were not different from control at any time through 14 days after treatment (Bruner et al., 1983). Alterations in Type I pneumocytes have not been reported for MCT-treated rats, but following treatment with MCTP the nuclei of Type I cells become enlarged and the cytoplasm swells and contains increased cytoplasmic organelles (Butler, 1970; Hurley and Jago, 1975). Observations of vascular responsiveness in isolated, perfused lungs also differ slightly following treatment with MCT and MCTP. While the pulmonary vasculature is hyperresponsive to vasoconstrictors early (day 4) but not later (day 7 or 14) after treatment with MCT (Gillespie et al., 1986), following treatment with MCTP vasoconstriction is enhanced in isolated lungs at days 7 and 14 (Hilliker and Roth, 1985a). Finally, while relatively low doses of either MCT or MCTP produce chronic pulmonary vascular injury and pulmonary hypertension, the effect of administration of larger doses which cause death within 2 days differs between MCT and MCTP. MCTP causes frank pulmonary edema and animals die from pulmonary complications (Hurley and Jago, 1975) while death shortly after MCT treatment is associated primarily with liver injury (Schoental and Head, 1955; Tuchweber et al., 1974).

c. Bioactivation of MCTP?

It does not appear that MCTP requires further bioactivation. Treatment with an inducer or an inhibitor of cytochrome P450 which does alter MCT toxicity did not alter the pneumotoxicity or RVE caused by MCTP (Bruner et al., 1986). Although this result does not rule out the possibility that

P450 isoenzymes or other enzyme systems unaffected by these agents may bioactivate MCTP, no evidence exists to suggest a role of such enzymes.

d. Development of MCTP toxicity

Bruner and coworkers (1983) outlined the time-course of biochemical and hemodynamic changes following a single intraveous injection of MCTP (5 mg/kg). There was a delay in the development or expression of injury such that 3 days after treatment no signs of major injury were apparent. By day 5 lavage fluid protein concentration and LDH activity were elevated in treated rats relative to controls, and these indices remained elevated through day 14. Pulmonary artery pressure did not increase until day 7, and at this time elevated wet lung weight and vascular leak were also evident (Bruner et al., 1983, 1986). Pulmonary artery pressure remained elevated through day 14, and RVE developed between days 10 and 14. This suggested that the right ventricle enlarges in response to sustained pulmonary hypertension.

D. Interest in MCT/MCTP Toxicity

1. As an environmental toxicant

PZA poisoning caused by animals grazing on PZA-containing plants is responsible for considerable livestock and, therefore, economic losses (Snyder, 1972; Huxtable, 1979). Human exposure is often fatal as well (Hill et al., 1951; McLean, 1970), therefore PZAs represent an environmental health problem.

2. As a pneumotoxic metabolite produced in the liver

MCT also represents a model compound for the study of toxicants which are bioactivated in the liver to metabolites which produce pulmonary toxicity.

3. As a model for human pulmonary vascular disease

Much of the pathophysiology described in MCT-treated rats is similar to that seen in humans with chronic pulmonary vascular diseases such as

primary pulmonary hypertension (PPH) and adult respiratory distress syndrome (ARDS). For this reason, the MCT-treated rats presents a good animal model for the study of these human diseases.

a. Primary pulmonary hypertension

PPH is pulmonary hypertension of unexplained etiology. A diagnosis of PPH is reached if three criteria are met: 1) right ventricular hypertrophy in the absence of other cardiac abnormalities; 2) elevated pulmonary arterial pressure and normal pulmonary wedge pressure; and 3) the absence at autopsy of evidence for any other etiology of pulmonary hypertension (Voelkel and Reeves, 1979). Pulmonary pathology very similar to that reported in MCT-treated rats has been described for patients with PPH. Intimal fibrosis, pulmonary arteritis, swollen ECs, platelet thrombi, and vessel occlusion have been observed (Walcott et al., 1970; Watanabe and Ogata, 1976; Voelkel and Reeves, 1979; Palevsky and Fishman, 1985; Reid, 1986). The pulmonary arterial media hypertrophies, the number of small vessels decreases, and ghost arteries appear in lungs of these patients. Plexiform lesions are considered a hallmark of PPH in man, and have also been observed in MCT-treated rats (Watanabe and Ogata, 1976). In addition, as in MCT-treated rats, alterations in respiratory mechanics are observed (Fernandez-Bonetti et al., 1983), and pulmonary extraction of biogenic amines is impaired (Sole et al., 1979).

Clinically, PPH presents a problem because signs and symptoms are non-specific and appear only after marked vascular alterations have developed. Therapy is largely ineffective, and the prognosis of these patients is quite poor (McLeod and Jewitt, 1986). Thus, monocrotaline provides a useful animal model to study a problematic human disease.

b. Adult respiratory distress syndrome

ARDS is a complication in people who suffer physical trauma, sepsis, or pneumonia (Bernard and Brigham, 1985). Early stages of ARDS are associated with increased vascular permeability due to leakage from capillaries and microvascular disruption, and this results in interstitial edema and alveolar flooding (Bernard and Brigham, 1985). EC injury is observed accompanied by compromised pulmonary extraction of 5HT (Morel et al., 1985), and pulmonary arterial pressures are elevated (Snow et al., 1982). Airway abnormalities are also evident, including destruction of Type I cells and decreased compliance (Bernard and Brigham, 1985). These changes are similar to those observed following administration of high doses of MCTP to rats (Plestina and Stoner, 1972; Hurley and Jago, 1975).

As ARDS progresses, changes seen are similar to those observed following treatment with relatively low doses of MCTP. Later stages of ARDS are associated with pulmonary hypertension and vascular remodelling (Snow et al., 1982). Medial thickness of small pulmonary arteries increases, and there is a trend toward a decrease in the external diameter of partially muscular arteries, suggesting that small, nonmuscular arteries are developing into partially muscular arteries (Snow et al., 1982).

Thus, the alterations associated with the early stage of ARDS are similar to those seen following high doses of MCTP, and alterations associated with later stages of ARDS are similar to those seen following modest doses of MCTP. Therefore, MCTP may be useful as a model of this human pulmonary vascular disease.

E. Problems Associated with Studying this Model

As with many animal models, there are a number of difficulties inherent in the study of MCT-induced pneumotoxicity. A few of them bear

mention here, in that they relate to experimental design and interpretation of results.

For example, the requirement for bioactivation of MCT introduces difficulties in interpreting the results of some studies in which "specific" drugs are used in the hope of uncovering a mechanism of action of MCT. If such a drug attenuates the toxicity of MCT, the question must be raised as to whether this protective effect can be attributed to the specific action of the drug or whether it is a consequence of inhibition of bioactivation of MCT to a toxic metabolite. To circumvent this problem, drug treatment studies have been performed in rats treated with the pyrrole metabolite, MCTP, which apparently does not require bioactivation.

Another difficulty arises from the complex nature of the vascular alterations caused by MCT or MCTP. Because these effects are numerous, and span several cell types and various cell functions, the mechanism of action likely involves the interaction of many cells and mediators. Therefore, studies in vivo or in intact organs where the pulmonary architecture is maintained will likely provide the most useful information.

Hayashi and coworkers (1979) demonstrated that retardation of growth by restriction of food intake attenuated MCT-induced pneumotoxicity and RVE. This phenomenon also presents a problem in studying the mechanisms of MCT's actions. Because of the subacute nature of the toxicity, any attempt to ameliorate the effects of MCT by co-treatment with a specific drug necessitates treatment with that drug and/or observation of animals for one week or longer. Many drugs, when given at effective doses for this length of time, reduce body growth by themselves, again raising the possibility that a protective effect may not be due to the specific action of the drug, but may be due instead to a non-specific effect of retarded growth.

Yet another problem derives from the observation that daily intraperitoneal injections of water decrease the right ventricular hypertrophic response to MCT (Langleben and Reid, 1985). This protective effect was attributed to a response to the stress of handling and injection, and emphasizes the need for appropriate controls in drug treatment studies.

Despite the problems associated with studying MCT's mechanism of action, it is a useful animal model for examining pulmonary vascular disease, and it can provide important information in this area.

III. Mechanisms of Action of MCT/MCTP

A. General

Although early studies of MCT or MCTP were aimed largely at describing the associated pathophysiology, more recent investigations have been designed to determine the mechanism by which MCT causes pulmonary vascular damage. Several avenues of investigation have arisen, and the evidence for or against these potential mechanisms will be described in this section. A number of fundamental questions remain to be answered, however. For example, does vasoconstriction contribute to increased pulmonary vascular resistance at any time during the development of pulmonary hypertension? Evidence suggests that in human pulmonary hypertension smooth muscle proliferation and vascular remodelling are the result of vasoconstriction (Voelkel and Reeves, 1979). In MCT-induced pulmonary hypertension, increases in pulmonary artery pressure have been reported to precede (Watanabe and Ogata, 1976) or to follow (Ghodsi and Will, 1981) the appearance of medial hypertrophy. Thus, this issue has not been clearly resolved. Another question pertains to what role, if any, vessel leak and the resulting pulmonary edema play in the development of pulmonary hypertension in this model. Increases in vessel leak are observed prior to RVE

(Valdivia et al., 1967; Sugita et al., 1983a), suggesting that increased vascular permeability could contribute to pulmonary hypertension.

Yet another curious issue arises from the observation of a 3-day delay following treatment with MCTP prior to the onset of major lung injury (Bruner et al., 1983). This is probably 24-48 hours after the disappearance of the pyrrole (Allen et al., 1972; Mattocks, 1972), and suggests that toxicity may somehow be mediated indirectly.

These and other unanswered issues serve to emphasize that the vascular changes following administration of MCT or MCTP are varied and complex. To search for a single answer or mechanism may be unrealistic. Rather, a less naive approach may be to attempt to uncover factors which contribute to the response, and this is the approach most investigators are now taking.

B. The Role of Angiotensin Converting Enzyme

An increase in lung ACE activity was observed one week after administration of MCT (Molteni et al., 1984). Although later in the development of toxicity lung ACE activity was depressed (Keane et al., 1982; Molteni et al., 1984; 1985), it was conceivable that the early increase in ACE activity would contribute to the development of pulmonary hypertension. ACE inactivates the vasodilator bradykinin and converts inactive angiotensin I to the vasoconstrictor angiotensin II, and an increase in these activities could produce enhanced vasoconstriction in the pulmonary vascular bed.

It has been reported that co-treatment with the ACE inhibitor Captopril attenuated the development of RVE and the ultrastructural changes in MCT-treated rats (Molteni et al., 1985). However, treatment with Captopril alone reduced the weight of the right ventricle, which could have contributed to

the decrease in RV/(LV+S) observed in MCT-treated rats co-treated with Captopril. In addition, body weight was significantly lower in Captopril-treated rats than in controls, and this non-specific effect to retard body growth may have contributed to protection against RVE. More importantly, however, Captopril did not decrease lung ACE activity at the dosing regimen used in this study, and did cause an increase in serum ACE activity. This finding suggests that inhibition of ACE was not responsible for the observed decrease in RVE. Similar protection has been obtained with nonsulfhydryl-containing ACE inhibitors at doses which did or did not reduce lung ACE activity (Molteni et al., 1986).

Therefore, the results of these studies do not present a convincing case that ACE activity is involved in the pathogenesis of MCT-induced pulmonary hypertension. Interestingly, Captopril did reduce the amount of hydroxyproline in lungs of MCT-treated rats, indicating a reduction in collagen content in these lungs and suggesting that an anti-fibrotic activity of Captopril may be responsible for its protective effect.

C. The Role of Collagen Synthesis

Molteni and coworkers (1985; 1986) also investigated the role of collagen synthesis in MCT-induced pulmonary injury by co-treating rats with penicillamine, an inhibitor of collagen maturation. Using hydroxyproline content as an index of fibrosis, these investigators demonstrated that penicillamine, at a dose which prevented the MCT-induced increase in lung hydroxyproline content, decreased the pulmonary ultrastructural changes caused by MCT treatment. The increase in wet lung weight and RVE due to MCT were not affected by co-treatment with penicillamine. These results suggest that collagen synthesis may contribute to MCT-induced ultrastructural alterations. However, penicillamine also functions as a copper chelator, and serum copper levels are elevated in patients with primary pulmonary hypertension (Ahmed and Sackner, 1985) and in

rats with pulmonary hypertension induced by a metabolite of MCT (our own observations). Thus, if copper is involved in the ultrastructural changes induced by MCT, penicillamine may alter this response through chelation of copper.

D. The Role of the Immune Effectors

Because the delay in toxicity and the histopathologic profile seen following treatment with MCTP were consistent with an immune response, it was hypothesized that immune effectors may be involved in MCTP-induced pneumotoxicity (Bruner, 1985). A possible role for both a cell-mediated and a humoral-mediated immune response was examined.

The effect of co-treatment with immunosuppressant agents was determined in MCTP-treated rats. Immunosuppression induced with an antiserum directed against rat lymphocytes did not alter the toxicity of MCTP. Co-treatment with the immunosuppressant drug cyclosporin A attenuated the lung injury and RVE caused by MCTP, however, rats co-treated with cyclosporin A lost weight relative to controls, so that the possibility that this partial protection was due to a retardation in growth cannot be ruled out. When lymphocytes from MCTP-treated rats were transferred into MCTP-treated rats, pneumotoxicity was not altered. The results of these experiments suggest that a cell-mediated immune response is not involved in MCTP toxicity (Bruner, 1985). MCTP also did not activate complement in vitro or in vivo, and depletion of complement did not attenuate MCTP-induced lung injury. Thus, it appears that immune effectors are not involved in the response to MCTP.

E. The Role of Phagocytic Cells

Following a single injection of MCT, large numbers of cells were observed in the alveolar space which, on the basis of functional and ultrastructural studies, were determined to be abnormal alveolar macrophages (Sugita et al., 1983b). The number of these cells increased with increasing doses of MCT and

with the degree of elevation of right ventricular systolic pressure and RVE. It was suggested that these abnormal alveolar macrophages may represent a marker of MCT-induced pulmonary hypertension (Sugita et al., 1983b).

One mechanism by which macrophages or other phagocytic cells could contribute to the response to MCT or MCTP is through generation and release of reactive oxygen species which could cause cell injury. The ability of cells recovered in the bronchoalveolar lavage fluid to generate the superoxide anion was examined in MCTP-treated rats (Dahm et al., 1986). The total number of macrophages was lower in the lavage fluid of treated rats compared to controls 5 days after treatment, but not at 7, 10, or 14 days after treatment. In the lavage of treated rats compared to controls, the total number of neutrophils was elevated at days 7, 10, and 14, the total number of eosinophils was increased at day 10 and the total number of lymphocytes was increased at day 14. Superoxide production by cells in the lavage fluid was decreased in MCTP-treated rats compared to controls at days 7, 10, or 14 (Dahm et al., 1986). This could mean that these cells had released all superoxide before or during the lavage procedure and were not capable of releasing any more superoxide after recovery. If superoxide were released in vivo (before collection of lavage fluid), this reactive oxygen species could contribute to the tissue injury observed in lungs of MCTP-treated rats.

F. The Role of Reactive Oxygen

The role of reactive oxygen in MCTP-induced pneumotoxicity was further examined by Bruner (1985). MCTP-treated animals were co-treated with drugs which either prevent the formation of reactive oxygen species or degrade or scavenge them once produced. Desferroxamine chelates iron, which is necessary for the formation of hydroxyl radicals through the Fenton reaction. Catalase

causes the breakdown of hydrogen peroxide, and dimethysulfoxide (DMSO) scavenges hydroxyl radicals. Toxicity due to MCTP was unaffected by co-treatment with either desferroxamine, catalase, or DMSO (Bruner, 1985). These results suggest that oxygen radicals are not involved in MCTP toxicity.

G. The Role of Leukotrienes

Leukotrienes (LTs) arise from the membrane phospholipid arachidonic acid by action of the enzyme 5'-lipoxygenase (Figure 2). LTs produce a variety of biological effects. LTC_4, D_4 and E_4 are now known to be what was once called slow reacting substance of anaphylaxis (SRS-A). In vitro these three LTs produce smooth muscle contraction in a variety of tissues (Creese et al., 1984; Sirois et al., 1985), reduce coronary blood flow (Hedqvist et al., 1983), and may stimulate release of cyclooxygenase metabolites of arachidonic acid (Creese et al., 1984; Sirois et al., 1985). In vivo LTC_4 and D_4 alter pulmonary compliance, vascular permeability, and pulmonary vascular resistance (Feddersen et al., 1983; Leffler et al., 1984). LTB_4 causes chemotaxis, aggregation, and adherence of granulocytes (Hoover et al., 1984). It is a less potent stimulator of smooth muscle than SRS-A LTs, but also causes release of cyclooxygenase products (Sirois et al., 1985). Due to the inflammatory response produced by MCT treatment, and the biological effects of LTs, LTs have been proposed to be mediators of the pneumotoxic response to MCT.

There is some evidence that LTs are elevated in lungs due to MCT treatment. SRS-A-like activity was observed in lavage fluid of lungs from rats one week after a single injection of MCT (Stenmark et al., 1985). SRS-A-like activity was not observed in lavage fluid of control rats. Lavage fluid of treated rats also contained greater concentrations of LTC_4 than controls, and the presence of LTB_4. Two or 3 weeks after a single injection of MCT, no SRS-A-like activity was observed in the lavage fluid of treated or control rats. At 3 weeks,

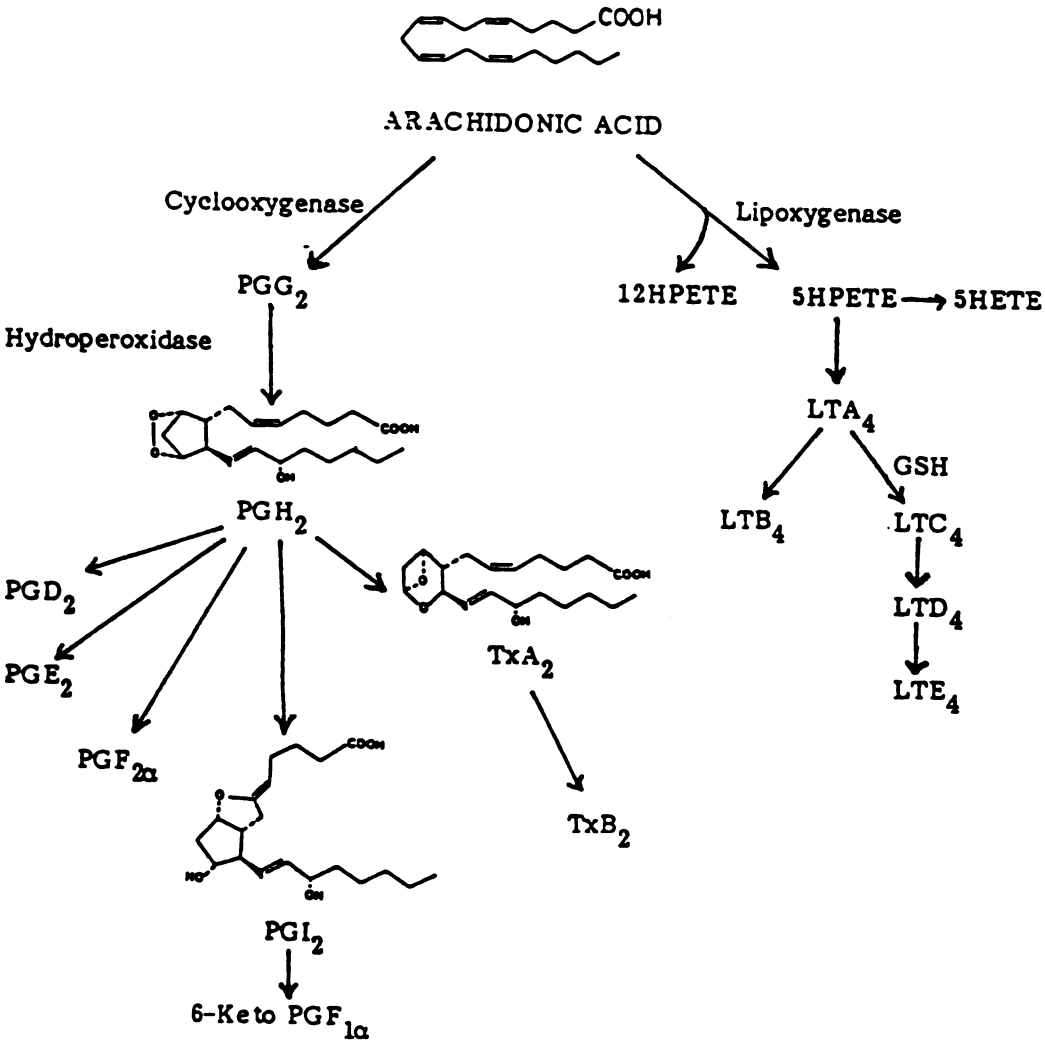

Figure 2. Pathways of arachidonic acid metabolism.

lavage fluid from treated rats inhibited contractile activity induced in isolated, guinea pig ileum by addition of exogenous LTC_4 or LTD_4. Homogenates of lungs from rats treated 3 weeks earlier showed SRS-A-like activity (Stenmark et al., 1985). These results suggested that LT synthesis may be activated by MCT treatment.

In rats treated 3 weeks earlier with MCT, co-treatment with diethylcarbamazine (DEC), which inhibits LT production (Mathews and Murphy, 1982), attenuated pulmonary hypertension and RVE (Stenmark et al., 1985). This is in contrast to the effect of co-treatment with DEC on the toxicity of MCTP (Bruner, 1985). DEC attenuated MCTP-induced lung injury at day 7, but did not alter pneumotoxicity or RVE at day 14. There are a number of possible explanations for the disparity in the results of the two studies. In the study with MCT, co-treatment with DEC began two days prior to treatment with MCT and continued through the end of the study, whereas co-treatment with DEC began 3 days after treatment with MCTP in the study of Bruner (1985). It is possible that the difference in dosing regimens could explain the different results obtained in these two studies. Another explanation could be that DEC was acting to protect against MCT toxicity in some manner unrelated to LT inhibition. For example, body weights were not reported in the study of DEC co-treatment of MCT-treated rats, and it is possible that DEC suppressed body weight gain. Alternatively, DEC could have inhibited biotransformation of MCT to its toxic metabolite.

DEC is not a specific inhibitor of LT synthesis, and more specific drugs that inhibit LT biosynthesis or act as LT receptor antagonists have not been used in this model. Therefore, additional experiments to determine if a role for LTs exists in MCT-induced pulmonary hypertension need to be performed.

H. The Role of Polyamines

Increases in lung polyamine concentrations are observed one week after treatment with a single injection of MCT (Olson et al., 1984b). Activities of the enzymes associated with polyamine biosynthesis are elevated in lungs early (day 1), and late (days 14-21) in the development of MCT toxicity (Olson et al., 1984a,b) and in ECs in culture exposed to MCT metabolites (Altiere et al., 1986a). Because polyamines may be essential for cell growth, it was hypothesized that they may be involved in the proliferation and hypertrophy of vascular smooth muscle observed following treatment with MCT. If the increase in vascular smooth muscle contributes to MCT-induced elevation of pulmonary arterial pressure, then polyamines may play a role in MCT-induced pulmonary hypertension.

Ornithine decarboxylase (ODC) is the rate-limiting enzyme in the biosynthesis of polyamines. Co-treatment with the ODC inhibitor, α-difluoromethylornithine (DFMO) attenuated the development of RVE and the increase in pulmonary artery pressure caused by MCT (Olson et al., 1984b). However, in this particular study, drug effectiveness was not confirmed, body weight data was not presented, and the effect of DFMO on bioactivation of MCT was not addressed. A subsequent study using the same protocol dealt with some of these issues (Olson et al., 1985). In this second study, DFMO prevented MCT-induced medial thickening, perivascular edema, and increased lung weight. DFMO partially suppressed the elevation of lung polyamine levels caused by MCT, and did not affect body weight gain. The production of Ehrlich-reactive products (pyrroles) from MCT in isolated livers or in liver slices was not altered by DFMO, suggesting that DMFO did not prevent the bioactivation of MCT. However, Ehrlich reactivity is positive for all pyrroles, and DFMO could have suppressed synthesis of a pyrrole metabolite responsible for lung injury without affecting total pyrrole production.

The results of these studies suggest that polyamines may somehow be involved in MCT-induced pneumotoxicity. However, as mentioned above, the issue of alteration of MCT bioactivation has not been sufficiently addressed. Furthermore, this conclusion is drawn from studies using only one inhibitor of polyamine biosynthesis. It is possible that DFMO may have other actions in addition to inhibition of ODC. Along this line, it is of interest that DFMO prevented all manifestations of MCT toxicity examined. This perhaps should not be surprising considering the multiplicity of actions that polyamines have in biological systems. Clearly, more studies should be done in this interesting and promising area. The case for polyamines would be strengthened if other drugs which inhibit polyamine synthesis or activity also protected against MCT-induced lung injury and pulmonary hypertension.

I. The Role of Blood Platelets

1. Evidence that platelets may be involved

A couple of observations initiated the thinking that blood platelets may be involved in the response to MCT. The first was that platelet-containing thrombi were seen in the pulmonary vessels of rats treated with MCT or MCTP (Merkow and Kleinerman, 1966; Chesney et al., 1974a; Hurley and Jago, 1975; Heath and Smith, 1978). The second was that, following treatment with MCT, a mild thrombocytopenia was observed (Hilliker et al., 1982). These two observations were consistent with the damaged ECs observed by electron microscopy (Turner and Lalich, 1965; Merkow and Kleinerman, 1966; Butler, 1970; Chesney et al., 1974a), and it was hypothesized that EC injury promoted platelet/vessel wall interaction. Activated or modified platelets could then in some way contribute to pulmonary hypertension.

This hypothesis was supported by the finding that antibody-induced thrombocytopenia attenuated the development of RVE in MCTP-treated

rats (Hilliker et al., 1984a). Rats made moderately thrombocytopenic (circulating platelet number approximately 15% of normal) from days 3-5 or from days 6-8 following a single treatment with MCTP developed less severe RVE at day 14 than rats with normal platelet numbers. Since RVE is thought to be a response to sustained increases in pulmonary artery pressure, this suggested that platelet depletion reduced MCTP-induced pulmonary hypertension. Lung injury assessed at day 14 was unaffected by platelet depletion. There are several possible explanations for this last result. One interpretation is that the platelet does not play a role in MCTP-induced pulmonary injury but rather is involved in the response to MCTP-induced injury (i.e., pulmonary hypertension). Alternatively, reducing the number of circulating platelets could delay MCTP-induced toxicity. Normally, major lung injury appears between days 4 and 8 whereas RVE is not evident until day 14 after MCTP treatment (Bruner et al., 1983). If thrombocytopenia were to delay the onset of toxicity by a few days, then at day 14 lung injury may still be apparent but RVE would not be. At any rate, there is some evidence to suggest that blood platelets may be involved in the pulmonary hypertensive response to MCTP.

No protective effect was observed if the period of platelet depletion was from days 0-2 following administration of MCTP. This suggests that platelets may not be involved in the early injury caused by MCTP, and that platelet involvement may not be a factor until several days after MCTP treatment.

2. Mechanisms by which platelets may be involved

There are a number of mechanisms by which platelets could be involved in MCTP-induced pulmonary hypertension. For example, platelets function by some ill-defined mechanisms to maintain normal, uninjured endothelium (Roy and Djerassi, 1972; Kitchens and Weiss, 1975), and an alteration of this function could conceivably contribute to increased pulmonary vascular pressures.

Alternatively, platelet-containing thrombi could occlude pulmonary vessels and increase pulmonary vascular resistance. Another possible mechanism is that platelets encountering damaged endothelial cells in the pulmonary vascular bed could become activated and release mediators which alter vascular tone or permeability or which stimulate smooth muscle cell proliferation. This last potential mechanism will now be further discussed.

3. Platelet mediators that may be involved

When platelets interact with an injured vessel wall they can become activated, meaning that they can adhere to the surface of the vessel, aggregate, and undergo a release reaction (Ratliff et al., 1979; Weiss, 1982). Intravascular platelet aggregation causes increased pulmonary vascular resistance, increased pulmonary vascular permeability, and alterations in respiratory function (Bø and Hognestad, 1972; White et al., 1973; Vaage et al., 1974, 1976). These responses are believed to be mediated by products of the release reaction. Mediators released by stimulated platelets include 5HT, arachidonic acid metabolites (such as 12HPETE, PGH_2, TxA_2), platelet-derived growth factor (PDGF), and platelet-activating factor (PAF).

a. 5-Hydroxytryptamine

Platelets accumulate 5HT by an active uptake process (Born et al., 1972; Talvenheimo et al., 1979) and most of this 5HT is stored in dense granules, although some extravesicular storage pools also exist (Tranzer et al., 1966; Holmsen et al., 1969; Costa et al., 1982; Given and Longenecker, 1985). 5HT released during platelet aggregation can cause vasoconstriction in a number of vascular beds (DeClerck and Van Nueten, 1982; Mullane et al., 1982; McGoon and Vanhoutte, 1984), including the lung (Rickaby et al., 1980; Tucker and Rodeghero, 1981). 5HT can also potentiate the response to other vasoconstricting agents (DeClerck and Van Nueten, 1982) and can increase vascular permeability

(DeClerck et al., 1984, 1985). Thus, 5HT could contribute to MCTP-induced pulmonary hypertension through vasoconstriction, potentiation of the action of other vasoconstrictors, or alteration of vascular permeability.

b. Arachidonic acid metabolites

Arachidonic acid (AA) is a 20-carbon fatty acid found most frequently esterified at the sn-2 position of membrane phosphoglycerides. AA is liberated from phospholipids by the action of phospholipase A_2 or phospholipase C. Free AA is metabolized by two major pathways: conversion by the enzyme cyclooxygenase produces prostaglandins (PGs) and thromboxanes (TXs); conversion by lipoxygenase enzymes produces hydroperoxyeicosatetraenoic acids (HPETEs), hydroxyeicosatetraenoic acids (HETEs), and leukotrienes (LTs) (Figure 2). Although small amounts of 12-HPETE are produced in platelets, the major pathway of AA metabolism in platelets is via the cyclooxygenase enzyme to produce the endoperoxides PGG_2 and PGH_2, which in turn degrade to form PGE_2, $PGF_{2\alpha}$, and TxA_2 (Needleman et al., 1976; Longenecker, 1985).

TxA_2 is the major AA metabolite synthesized by platelets. By inhibiting adenyl cyclase, and thereby decreasing platelet cyclic AMP, TxA_2 promotes platelet aggregation (Hamberg et al., 1975b; Meyers et al., 1979; Parise et al., 1984; Longenecker, 1985). In addition, TxA_2 causes vasoconstriction (Hamberg et al., 1975b; Svensson et al., 1977; Farrukh et al., 1985). Therefore, by virtue of its functions to promote platelet aggregation and to stimulate vascular smooth muscle to contract, TxA_2 may contribute to MCTP-induced pulmonary hypertension.

The pro-aggregatory and vasoconstrictive activities of TxA_2 are opposed in vivo by the actions of prostacyclin (PGI_2), synthesized largely by vascular endothelial cells (Moncada et al., 1976; Weksler et al., 1977; Tateson et al., 1977; MacIntyre et al., 1978). It has been proposed that the

balance of TxA_2 and PGI_2 is an important factor in vascular homeostasis (Gryglewski et al., 1976; Bourgain et al., 1982; Flynn and Demling, 1982; Saldeen and Saldeen, 1983). Endothelial cell injury associated with MCTP may alter PGI_2 synthesis, thereby allowing greater expression of the vasoconstrictive activity of TxA_2. In addition, normal endothelial cells may "steal" platelet-derived PGH_2 as a substrate for PGI_2 synthesis (Gorman, 1979), and if this function is suppressed due to EC injury following treatment with MCTP, increased TxA_2 synthesis by the platelet might result. This presents another mechanism by which TxA_2 could contribute to MCTP-induced pulmonary hypertension.

c. Platelet-derived growth factor

Platelet-derived growth factor (PDGF) is a polypeptide contained within platelet alpha granules and released during platelet activation (Deuel and Huang, 1984). PDGF stimulates DNA synthesis and proliferation of a number of cells including fibroblasts and smooth muscle cells. Since vascular smooth muscle proliferation and extension into normally nonmuscular vessels is a feature of MCTP-induced pulmonary hypertension, PDGF may contribute to the response to MCTP by stimulating smooth muscle cell proliferation.

d. Platelet-activating factor

Platelet-activating factor (PAF; acetylglycerylether phosphorylcholine) is a lipid mediator that aggregates platelets (Chignard et al., 1979; Macconi et al., 1985) and polymorphonuclear neutrophils (Camussi et al., 1980). PAF also causes smooth muscle contraction, pulmonary edema, and alterations in pulmonary vascular permeability (Findlay et al., 1981; Mojarad et al., 1983; Lichey et al., 1984; Benveniste and Chignard, 1985). These actions of PAF might contribute to MCTP-induced pulmonary hypertension. PAF caused pulmonary hypertension and edema in isolated rabbit lungs perfused with human platelets,

and this response was mediated by PAF-induced release of TxA_2 (Heffner et al., 1983).

J. Summary

In summary, it appears that MCT or MCTP produce pneumotoxicity and pulmonary hypertension by a mechanism(s) which likely involves multiple mediators. One promising area of research is that of the contribution of polyamines to MCT-induced toxicity, and this area warrants further investigation. The remainder of this thesis will focus on studies which examine a possible role for the platelet in MCTP-induced pulmonary hypertension.

IV. Specific Aims

Platelets appear to be involved in the pulmonary hypertensive response to MCTP by an undetermined mechanism. It is apparent that a number of platelet mediators could contribute to pulmonary hypertension due to MCTP. The hypothesis that platelet-derived mediators contribute to MCTP-induced pulmonary hypertension was tested by performing experiments to:

A. Determine the dose/response relation for MCTP.

B. Describe the histopathology of the development of MCTP-induced pneumotoxicity.

C. Examine the influence of diet restriction on MCTP-induced cardiopulmonary toxicity.

D. Determine the effect of thrombocytopenia on MCTP-induced pulmonary hypertension.

E. Examine the role of 5HT in MCTP-induced cardiopulmonary toxicity.

F. Examine the role of TxA_2 in MCTP-induced cardiopulmonary toxicity.

MATERIALS AND METHODS

I. Animals

Respiratory disease-free, male, Sprague-Dawley rats (CF:CD(SD)BR) (Charles River Laboratories, Portage, MI) were used in these studies. They were housed on corn cob bedding in plastic cages under conditions of controlled temperature and humidity. An alternating 12-hour light/dark cycle was maintained. Whenever possible, cages were kept in an animal isolator (Contamination Control, Inc., Lansdale, PA) so that the rats breathed only HEPA-filtered air.

With the exception of rats used in the diet restriction study, all animals were allowed free access to food (Wayne Rodent Blox^{R}, Continental Grain Company, Chicago, IL) and water.

An adult, female Nubian goat was used for the preparation of anti-rat platelet antibody. The goat was kept outdoors in a small fenced-in pasture on which there was also provided a wooden shelter. Grass and water were freely available.

II. Diet Restriction

Rats were housed individually (one per cage) for this study. Standard rat chow was ground to a powder and was provided in small glass jars placed inside the cages. Rats were allowed to eat ad libitum (average daily food consumption was 22 g/rat) or were restricted to 9 g food/rat/day. Water was provided ad libitum for all rats.

III. Monocrotaline Pyrrole (MCTP)

A. Synthesis of MCTP

MCTP was synthesized from monocrotaline (MCT) (TransWorld Chemicals, Washington, D.C.) via an N-oxide intermediate as described by Mattocks (1969). The MCTP isolated from the synthesis procedure has Ehrlich activity (an indication of pyrrole moieties) (Mattocks and White, 1971), and a structure consistent with MCTP as determined by mass spectrometry (Mattocks, 1969; Culvenor et al., 1970; Bruner et al., 1986) and nuclear magnetic resonance (Bruner et al., 1986). MCTP was stored under nitrogen in the dark at -20°C.

B. Treatment with MCTP

MCTP was dissolved in N,N-dimethylformamide (DMF) at appropriate concentrations so that the volume injected was 0.5 ml/kg to achieve the desired dose. Rats were treated via the tail vein with DMF or with MCTP at doses ranging from 2-5 mg/kg.

IV. Assessment of Cardiopulmonary Injury

A. Bronchoalveolar lavage

The trachea of rats anesthetized with sodium pentobarbital (50 mg/kg, i.p.) was cannulated with a blunted 18 gauge hypodermic needle. The abdomen and thorax were then opened and the lungs were carefully removed. A known volume of room temperature saline (0.9%) was instilled into the airway and then removed. This procedure was repeated once, and the recovered lavages were combined. The volume of saline instilled was determined by multiplying the mean body weight (in kg) of all rats to be killed on a given day (control and treated) by 23 ml/kg (Mauderly, 1977). The lavage fluid was spun in a centrifuge (600 x g, 10 minutes), and the activity of lactate dehydrogenase (LDH) was determined in the cell-free supernatant on the day the lungs were lavaged. LDH activity was

measured spectrophotometrically according to the method of Bergmeyer and Bernt (1974) and was quantified as the conversion of the cofactor NADH to NAD^+ as pyruvate substrate was converted to lactate.

Protein concentration was determined in the cell-free lavage fluid using the method of Lowry and coworkers (1951). Bovine serum albumin was used as the protein standard.

B. Pulmonary sequestration of radiolabelled protein as a marker of pulmonary vascular leak

Pulmonary vascular leak was assessed as the accumulation of ^{125}I in the lungs following an intravenous injection of ^{125}I-labelled bovine serum albumin (^{125}I-BSA), using a modification of the method of Johnson and Ward (1974).

Rats were given an intravenous injection of ^{125}I-BSA (0.2 ml, 1.0 mg BSA/ml) containing approximately 400,000 cpm. Four hours later, rats were anesthetized with sodium pentobarbital, and 500 units of heparin (in 0.5 ml saline) were injected into the inferior vena cava. One minute later, one ml of blood was withdrawn and placed in a tube for determination of radioactivity (^{125}I-blood) (Tracor 1185 series Gamma Counter, Chicago, IL, or Micromedic Automatic Gamma Counter, Horsham, PA). The trachea was cannulated as described above for bronchoalveolar lavage, and the pulmonary artery was cannulated with a saline-filled catheter (PE 190). The lungs and heart were removed from the thorax and the left atrial appendage was cut. The lungs were periodically ventilated with a small volume of room air while 10 ml of saline were gently perfused through the pulmonary arterial cannula to clear blood from the vasculature. If lavage fluid LDH activity or protein concentration was to be determined, the lungs were lavaged at this point as described above. The lungs were trimmed from the trachea and connective tissue, rinsed with saline, blotted dry and placed in tubes for determination of radioactivity. If the lungs had been lavaged, one ml of lavage fluid was also placed in a tube for determination of radioactivity. The

total radioactivity in the lung (^{125}I-lung) was the radioactivity in the lung tissue plus, where applicable, the radioactivity removed in the lavage fluid (cpm/ml lavage fluid x ml lavage fluid recovered). An increase in the ratio ^{125}I-lung/^{125}I-blood was considered to indicate vascular leak.

C. Lung weight

Wet lung weight was determined as the difference in the weight of the lung plus connective tissue prior to bronchoalveolar lavage and vascular perfusion and the weight of the connective tissue after the lung was trimmed away.

Dry lung weight was determined after lungs, placed in pre-weighed vials and kept in a drying oven (92°C), reached a constant weight.

D. Pulmonary artery pressure

Pulmonary artery pressure (PAP) was measured in anesthetized rats. The distal end of a 3.5 French umbilical vessel catheter was warmed and bent to approximately a 45° angle. The catheter was introduced through the right jugular vein, carefully advanced into the right ventricle, and then gently manipulated into the pulmonary artery (Stinger et al., 1981). Pressure was measured with a Statham P23ID pressure transducer and was recorded on a Grass Model 7 polygraph. Location of the catheter was determined by characteristic pressure tracings.

E. Right ventricular enlargement

Right ventricular enlargement (RVE) was assessed as an increase in the ratio of the weight of the right ventricle to the weight of the left ventricle plus septum (RV/(LV+S)) (Fulton et al., 1952). The heart was removed, and the atria were carefully trimmed away. The wall of the right ventricle was then trimmed free of the left ventricle plus septum, and each tissue was weighed separately.

F. Other indices of injury

Blood urea nitrogen (BUN) was determined using a standard diagnostic kit (Urea Nitrogen No. 535; Sigma Chemicals, St. Louis, MO). Serum glutamic oxalacetic transaminase (SGOT) was also determined using a standard diagnostic kit (aspartate aminotransferase No. 505; Sigma Chemical, St. Louis, MO).

V. Histopathology

A. Fixation procedure

Following measurement of PAP in anesthetized rats, an abdominal incision was made and 500 Units of heparin (in 0.5 ml saline) were injected into the inferior cava. The trachea was cannulated (PE 210 tubing) and the thorax was opened. The pulmonary artery was cannulated (PE 190 tubing), the heart was removed, and the lungs were carefully excised from the thorax. The lungs were gently inflated with room air several times to distend atelectic portions. A modified Karnovsky's fixative (1% glutaraldehyde; 2% paraformaldehyde; 0.1 M cacodylate buffer; pH=7.4) was infused at constant pressure into the pulmonary artery from a height of 32 cm H_2O, and into the trachea from a height of 26 cm H_2O, for a minimum of 15 minutes. Sections of lung tissue (2-3 mm) were then cut and fixed in Karnovsky's fixative for an additional 4 hours at 4°C. Lung sections were rinsed twice in cold cacodylate buffer (0.2 M, pH=7.4) and were stored for future processing.

B. Tissue processing and staining

A longitudinal section (2-3 mm thick) of the left lung lobe, as well as sections from areas with obvious lesions or from areas randomly selected, were embedded in paraffin. Sections (4 μm) of paraffin embedded tissues were stained with hematoxylin and eosin stain (H and E).

Sections from areas of the lungs with gross lesions or from the right caudal lobe were selected for glycol methacrylate (plastic) embedding. These tissues were cut (1-2 μm) and were stained with toluidine blue and modified Gill's H and E stains.

VI. Preparation of Goat Anti-Rat Platelet Antibody

A. Preparation of a pre-immune (control) serum

Prior to exposure to the platelet membrane antigen, the goat was bled on several occasions to obtain control serum (CS). Blood (approximately 300 ml) was collected from the external jugular vein using a 16 gauge needle attached to PE260 tubing. The blood was allowed to clot at 37°C for 2 hours, after which it was spun (1000 x g) in a centrifuge for 10 minutes. The supernatant was incubated at 56°C for 45 mintues to deactivate complement, then cooled in an ice bath to approximately room temperature before being spun again as described above. The supernatant (CS) was then kept frozen (-2°C) until use.

B. Preparation of antigen: Rat platelet membranes

Retired breeder donor rats were anesthetized lightly with ether, and blood (10-20 cc) was withdrawn from the abdominal aorta into syringes containing 3.8% sodium citrate. The citrate volume was then adjusted so that the final concentration in the blood was 0.38%. The pooled blood was spun (150 x g, 10 minutes) and the platelet-rich plasma (PRP) was removed with a pipette, being careful to avoid red blood cells and the "buffy coat" in the process. This step was repeated until no more PRP could be collected.

The PRP was then spun (600 x g) for 10 minutes to obtain a platelet pellet. This procedure was repeated until the supernatant was exhausted of platelets as determined by the lack of appearance of a pellet. The platelet pellets were resuspended and washed 3 times in 0.01% EDTA in saline (1 ml), and were

pooled. The pooled platelet pellet, suspended in a minimum of 0.01% EDTA, contained 1.0×10^7 pt/µl. No red or white blood cells were seen when the suspension was examined microscopically. This suspension was frozen and thawed twice to facilitate lysing the platelet membranes. After the second thawing, the suspension was sonicated using a Sonicator Cell Disruptor (Heat Systems-Ultrasonics, Inc., Plainview, NY) to further disrupt the platelets. This suspension had less than 200 pt/µl. The protein concentration was determined, and the remaining suspension was diluted with 0.01% EDTA to adjust the protein concentration to 20 mg/ml (corresponding to approximately 6×10^6 pt/µl). Before use, an equal volume of Freund's complete adjuvant was emulsified with the platelet membrane solution using a homogenizer.

Antigen was prepared on a number of occasions by the above procedure, each time attempting to achieve approximately the same final concentration of protein.

C. Preparation of goat anti-rat platelet serum

The goat's back was shaven, and a total of 1 ml of the platelet membrane antigen emulsification was injected intradermally into 15-20 spots along her back (approximately 0.05 ml/injection). One week and two weeks later she received another 1 ml of antigen in an identical fashion ("booster"). Ten days later, blood was withdrawn from the external jugular vein and anti-platelet serum (PAS) was prepared from the blood as described above for CS.

The goat was bled 5 more times, each time preceded by a booster injection of antigen. PAS collected on different days was stored separately, and the potency of each was determined in vivo as described below.

D. Absorption of sera with red blood cells

To reduce any toxic effects of the sera, both the CS and the PAS were absorbed to washed, rat red blood cells (RBC's) before use. Blood was collected

from the abdominal aorta of sodium pentobarbital-anesthetized donor rats into syringes containing 1000 Units of heparin (in 1 ml saline). The blood was centrifuged (600 x g for 10 min) and the plasma discarded. The RBC's were then washed with saline 3 times. Washed RBC's were added to the serum (1 ml RBC's/5 ml serum) and this was kept at room temperature for one hour. After removing the RBC's by centrifugation (600 x g for 10 min), the absorption procedure was repeated once. Absorbed antisera was kept frozen until immediately before use.

E. Efficacy of PAS in vivo

The efficacy of the PAS was determined in vivo. Rats were treated with one of several doses of PAS intraperitoneally to establish a dose which produced the degree of thrombocytopenia desired. Once established, this dose was given (i.p.) to several rats immediately after taking blood from the tail for a baseline platelet count. Blood was then taken at various times after administration of the PAS, and platelet count and hematocrit were determined. Total white blood cell counts and differential white cell counts were also determined 12 hours after treatment with the PAS.

VII. Cell Counting

Platelet number was determined in platelet-rich plasma or in whole blood collected from the abdominal aorta or from the tail. Blood collected from the abdominal aorta was diluted with sodium citrate in a plastic syringe during collection so that the final concentration of citrate in blood was 0.38%. When blood was sampled from the tail, approximately 0.45 ml was allowed to drip into a polypropylene tube containing 50 µl of 3% trisodium EDTA in saline. Aliquots of blood or platelet-rich plasma were diluted with ammonium oxalate buffer using a Unopette^{R} diluting system (Becton Dickinson, Rutherford, NJ). Platelets were

counted in a Neubauer hemacytometer using phase contrast microscopy (Brecher and Cronkite, 1950).

White blood cells were counted in whole blood as described above for platelets. Differential cell counts were determined from Wright's stained-smears by counting and identifying 100-200 cells.

VIII. Platelet Aggregation

A. Blood collection

Rats were lightly anesthetized with ether, and blood was collected from the abdominal aorta. Plastic syringes containing 3.8% sodium citrate were used, and the volume of citrate was adjusted to a final blood:citrate ratio of 9:1.

B. Preparation of platelet-rich plasma and platelet poor plasma

Platelet-rich plasma (PRP) was prepared by one of two methods. In the first method, blood samples were spun 3 times at 150 x g for 5 minutes in an IEC HN SII centrifuge. After each centrifugation, the PRP was transferred to a clean polypropylene tube and the PRP fractions for each blood sample were pooled. In the second method, PRP was collected after each of two 1-minute centrifugations of whole blood at 600 x g in an IEC HN SII centrifuge. Platelet number in the PRP collected by either manner was similar.

Platelet-poor plasma (PPP) was collected after a 20-minute centrifugation (600 x g) of the remaining blood. Autologous PPP was used to adjust the platelet count of the PRP to approximately $10^6/mm^3$ for platelet aggregation studies.

C. Platelet aggregation

PRP (0.5 ml) was transferred to silanized glass cuvettes, and was allowed to settle for 30-60 minutes at room temperature before aggregation. Platelet aggregation was measured as an increase in light transmittance through a

cuvette of PRP (Born and Cross, 1963) using a dual channel platelet aggregometer (Payton Assoc., Buffalo, NY) and was recorded on an Omniscribe Chart recorder. Unstimulated PRP was used to set 0% transmission, and autologous PPP was used to set 100% transmission. Cuvettes containing PRP were inserted into the aggregometer and were allowed to equilibrate for 2 minutes (37.5°C, stirred at 900 rpm) before addition of the aggregating agents. Arachidonic acid (1.5 mM; sodium arachidonate, BioData Corp., Hatsboro, PA) was used as the stimulus for aggregation. Three aspects of platelet aggregation were recorded: the maximum aggregation (as a percent of the difference in light transmittance through PPP and unstimulated PRP), the rate of aggregation (slope = % aggregation/min, taken during the linear portion of the aggregation tracing); and the delay to aggregation (the time required for the shape change). These are illustrated in Figure 3.

D. Determination of platelet 5-hydroxytryptamine

Blood was collected from ether-anesthetized rats into syringes containing sodium citrate (0.38% final concentration) and pargyline HCl (0.1 mM final concentration; Abbott Labs, Abbott Park, IL). Platelet-rich plasma (PRP) was prepared according to the second method described above. An aliquot of the PRP was taken for determination of platelet number. Another aliquot (100 µl) was transferred to a microcentrifuge tube and was spun in an Eppendorf^{R} microcentrifuge for 5 minutes. HCl (2.5 N) was added to an aliquot of the supernatant to adjust the pH to 2.5, and this platelet-poor plasma was reserved for determination of 5-hydroxytryptamine (5HT). The pellet was washed with 1 ml of 0.01% Na_2EDTA containing 0.1 mM pargyline HCl, and then was resuspended in 1 ml of a phosphate buffer containing 0.1 mM pargyline HCl (0.05 M phosphate, 0.03 M citrate, 15% methanol, pH=2.7). The platelets were disrupted by persistent sonication (Sonicator Model W-220F, Ultrasonics, Inc., Plainview, NY). The platelet membrane solution was then spun in a microcentrifuge for 5 minutes, and

Figure 3. A typical aggregation curve depicting platelet aggregation parameters measured and points during aggregation at which the reaction was stopped and TxB_2 was determined. AA = aggregating agent. The extent of aggregation is calculated as a percentage of the difference in light transmittance in PPP (100%) and unstimulated PRP (0%). The rate of aggregation is expressed as % aggregation/minute, and the delay is expressed in minutes. a = unstimulated PRP (before addition of AA). b = half-maximal aggregation. c = maximal aggregation.

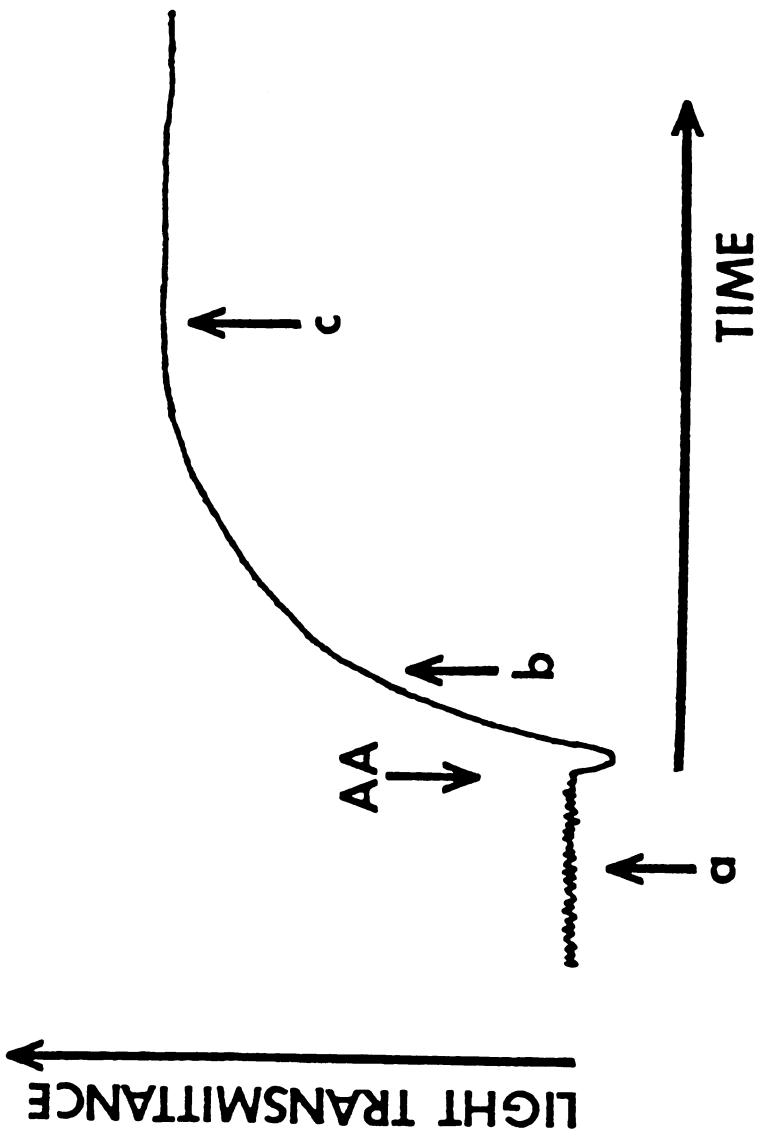

Figure 3

the supernatant was frozen until determination of 5HT by high performance liquid chromatography (HPLC). The protein concentration of the platelet pellet membrane was determined by the method of Lowry and coworkers (1951).

5HT was measured with an HPLC-electrochemical detection (HPLC-EC) system (Shannon et al., 1986). Fifty microliters of the sample supernatant were injected onto a C_{18} reverse phase column (4.6 mm i.d. x 25 cm; 5 µm spheres, Biophase ODS, Bioanalytical Systems, Inc., West Lafayette, IN) which was protected by a precolumn cartridge filter (10 µm spheres, 4.6 mm i.d. x 3 cm, Bioanalytical Systems, Inc.). The HPLC column was coupled to an electrochemical detector (LC4A, Bioanalytical Systems, Inc.) equipped with a TL-5 glassy carbon electrode set at a potential of +0.75 V relative to a Ag/AgCl reference electrode. The HPLC mobile phase (pH=2.7) consisted of 0.1 M citrate phosphate buffer, 0.1 mM EDTA, 1.5 mM sodium octyl sulfate and 25% methanol. Separations were performed with a flow rate of 0.8 ml/min and a pressure of approximately 2400 psi. The amount of 5HT in a sample was determined by comparing sample peak heights measured by a Hewlett-Packard 3390A Integrator (Hewlett-Packard, Avondale, PA) with standards that were run each day. The limit of sensitivity was 20 pg.

E. Determination of platelet-derived thromboxane B_2

Blood was collected from rats treated 1, 4, 7, or 14 days earlier with MCTP. PRP was collected according to the first method described above, and aggregation was induced with arachidonic acid (1.5 mM). The aggregation response was terminated by addition of 100 µl of 2 N HCl to the PRP. The PRP was then transferred to a 3 ml conical microcentrifuge tube and was spun in an microcentrifuge for 5 minutes. The supernatant was transferred to a clean tube and was frozen until determination of TxB_2 by radioimmunoassay (RIA).

TxB_2 generation was determined in unstimulated PRP, and in PRP which was at half-maximal or maximal aggregation following stimulation with AA. This is illustrated in Figure 3.

F. Effect of MCTP in vitro on platelet aggregation and TxB_2 generation

The effect of MCTP added in vitro to PRP was determined. Blood was collected from untreated rats and PRP was prepared as described above. DMF (1 µl) or MCTP (31-500 µg) in 1 µl of DMF was added to the PRP 1 minute prior to addition of AA. The concentration of TxB_2 in the PRP was determined at maximal aggregation.

IX. Isolated, Perfused Lungs

A. Surgery

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the trachea was cannulated with PE240 tubing. The abdomen was opened, and 500 U of heparin in 0.5 ml saline were injected into the inferior vena cava and allowed to circulate for approximately 1 minute. The diaphragm was cut along the abdominal wall, and then the ribcage was cut while carefully avoiding the lung tissue. The thymus was discarded, and the pulmonary artery was cannulated just above the right atrial appendage. The cannula used was made of PE190 tubing that was filled with saline and was then plugged. The heart was removed by cutting just above the ventricles, and was set aside for determination of RVE (RV/(LV+S)). The lungs were carefully removed from the thorax, and were inflated and deflated several times to prevent atelactasis. They were then transferred to the perfusion apparatus.

B. General perfusion procedure

Two different perfusion apparatuses were used. The one depicted in Figure 4 was used when the lungs were perfused with a buffer. The chamber in

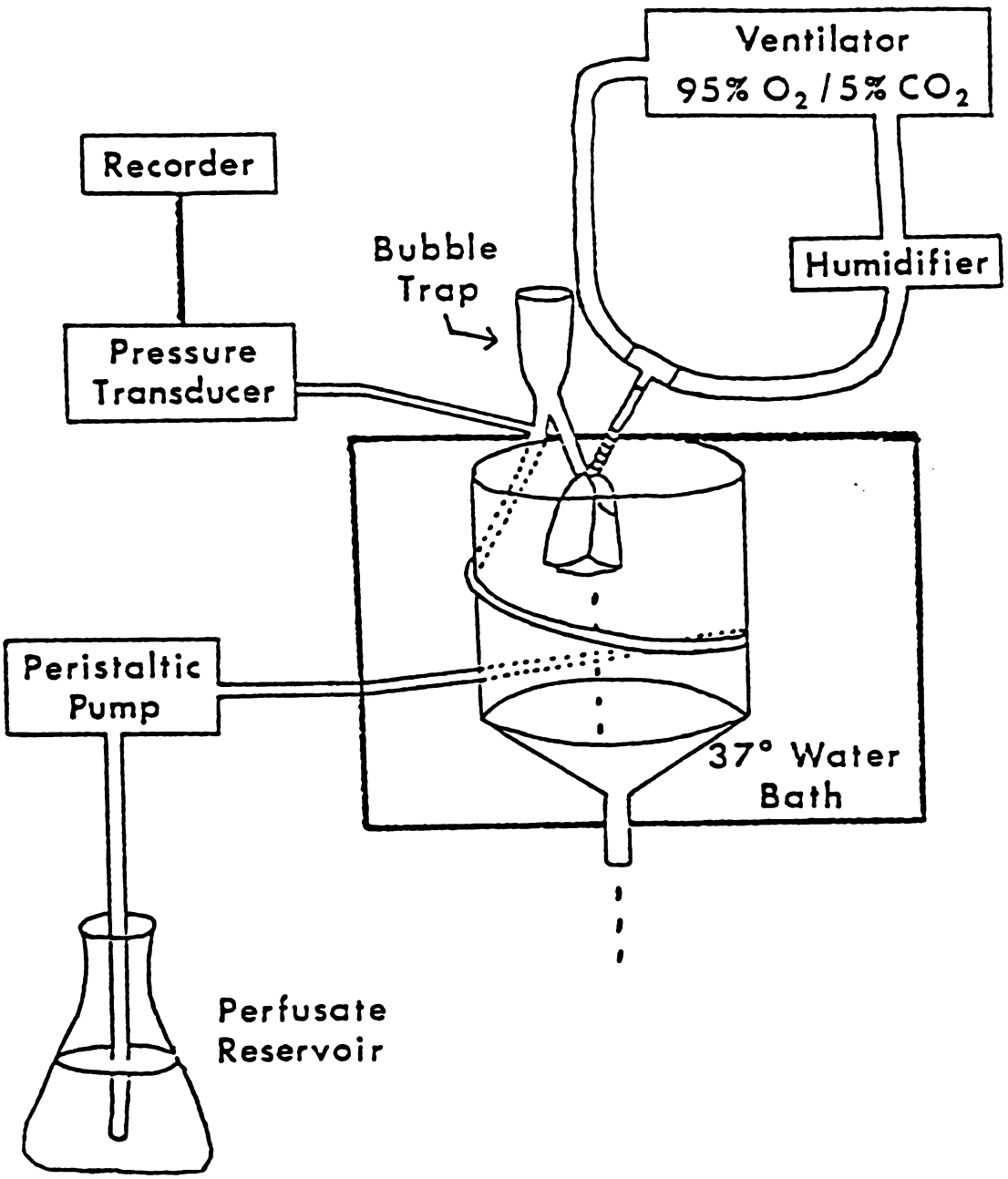

Figure 4. Diagram of isolated perfused lung preparation.

which the lung was suspended by the tracheal and pulmonary arterial cannulae was surrounded by water at 37°C. The perfusion medium, kept cold in an ice bath outside of the apparatus, was warmed as it circulated through the tubing in the water jacket. When blood was used as the perfusion medium, the perfusion apparatus consisted of a chamber maintained at 37°C, and the blood perfusate was stored in the chamber throughout the perfusion. The lungs were prevented from drying in the heated chamber by surrounding them with a "curtain" of moistened gauze pads.

Perfusion medium was pumped at a constant flow through the pulmonary artery and, after exiting the vasculature through the pulmonary vein, was either allowed to drip back into the perfusion medium reservoir (recirculating system) or was collected or discarded (single-pass system). When blood was used as the perfusion medium, a silk filter was inserted between the pump and the lung to prevent thrombi from reaching the lung.

For some experiments, the lungs were statically inflated with 2-3 ml of room air. For other experiments, the lungs were ventilated with warmed, humidifed 95% O_2/5% CO_2 using a small animal respirator (Mallard Medical Co., Irvine, CA). Inspiratory pressure was 13-16 cm H_2O, and a positive end-expiratory pressure of 2-3 cm H_2O was maintained.

Inflow perfusion pressure was monitored with a Statham P23ID pressure transducer and was recorded on a Grass Model 7 polygraph.

X. Effect of Ketanserin on the Vascular Response to 5HT in Isolated, Perfused Lungs

Rats to be used as blood donors or lung donors were treated with MCTP (4 mg/kg) and were co-treated with ketanserin or its vehicle, distilled water, as described below. Fourteen days after treatment with MCTP, lungs from rats treated with ketanserin or water were perfused with blood from rats treated with

ketanserin or water, respectively. Perfusions were performed 10-12 hours after the final dose of ketanserin or vehicle. Blood donors were lightly anesthetized with ether, and blood was withdrawn from the abdominal aorta into syringes containing heparin (final concentration = 1 U/ml). Blood was stored in the perfusion chamber until the perfusion. A 30 ml reservoir and a recirculating system was used. A gentle stream of humidifed 95% O_2/5% CO_2 was passed over the blood to maintain pH. In addition, during the perfusion pH was monitored, and Na_2CO_3 (0.5 M) or NH_4Cl (0.01 M) was added to the blood as appropriate to maintain pH between 7.35 and 7.45.

A 22 gauge needle, inserted through the stopper in the bubble trap close to the pulmonary arterial cannula, was attached to a 3-way stopcock and was used for delivery of drugs. Lungs were perfused for 10 minutes at 10 ml/min, then for an additional 10 minutes at 20 ml/min. After this equilibration period, saline (0.2 ml), angiotensin II (AII; Ciba-Geigy Corp., Summit, NJ, 0.5 µg) and 5HT (creatinine sulfate complex, 50 µg) were infused into the pulmonary artery in that order. After injection of each drug, an additional 0.2 ml saline was injected. At no time did saline produce a change in perfusion pressure, and, following infusion of AII, pressure was allowed to return to baseline before infusion of 5HT. The perfusions were terminated 5 minutes after injection of 5HT.

XI. Prostanoid Release in Isolated, Buffer-Perfused Lungs

A. Day 7 after MCTP treatment

Lungs from rats treated 7 days earlier with MCTP (4 mg/kg) were perfused at 10 ml/min in a single pass system using Krebs-bicarbonate buffer containing 4% bovine serum albumin (BSA) (Fraction V, Miles Biochemicals, Inc., Elkhart, IN). The lungs were perfused for 30 minutes and ventilated as described above. Effluent samples were collected periodically into polypropylene tubes

containing indomethacin (final concentration = 100 μM) and samples were kept frozen (-70°C) until determination of prostanoid concentrations.

In one experiment, lungs from a separate group of rats not treated with MCTP, but treated 1-2 hours earlier with indomethacin (10 mg/kg, i.p.) were perfused in a similar manner with perfusion medium containing indomethacin (0.5 mM).

B. Day 14 afer MCTP treatment

Lungs from rats treated 14 days earlier with MCTP, and lungs (from untreated rats) used to determine the concentration-response relation to arachidonic acid, were perfused at a flow of 8 ml/min in a non-recirculating system. These lungs were pre-perfused for 15 minutes with a Krebs-bicarbonate buffer containing 4% BSA. During this period the lungs were ventilated as described above. At time 0 (t = 0), the perfusate was switched to a BSA-free, Krebs-bicarbonate buffer, and the lungs were statically inflated with 2-3 cc room air. After a 10-minute stabilization period to wash out any remaining BSA, effluent samples were collected every minute as described above. After an additional 3 minutes (at t = 13 minutes), arachidonic acid (AA), prepared as described below and at the concentration indicated, was infused directly into the pulmonary artery at 0.1 ml/min. The AA was delivered from a 5 cc syringe using a syringe pump (Model 341, Orion Research Inc., Cambridge, MA). PE20 tubing connected the syringe to a T-connection at the bubble trap and was also fitted from the T-connection through the stopper in the bubble trap to the pulmonary arterial cannula.

The effect of edema formation on the release of prostanoids was determined in isolated, perfused lungs from untreated rats. The left atrium was cannulated and outflow pressure was elevated to approximately 8 cm H_2O. The lungs were perfused with a BSA-free buffer at a flow rate of 40 ml/min until they

appeared to have taken on fluid. At that time, outflow pressure was returned to 0 cm H_2O, and the flow was decreased to 8 ml/min. After 10 minutes, effluent samples were collected every other minute for 12 minutes.

C. Preparation of arachidonic acid for infusion into isolated, perfused lungs

Arachidonic acid was obtained from Sigma Chemical Company (St. Louis, MO) as the free acid. It was dissolved in absolute ethanol and stored frozen under nitrogen. Just prior to use, NaOH was added to convert it to the sodium salt, and appropriate dilutions were made with saline.

Purity of the arachidonic acid solution used in the perfusions was checked periodically by thin-layer chromatography (TLC). High performance silica gel plates (Whatman LHP-K) and a solvent system of ethyl acetate/acetic acid (99/1, v/v) were used. The plates were developed with iodine vapors and only one spot (average retention factor of 0.62 ± 0.3) was observed.

XII. Prostanoid Release in Blood-Perfused Lungs

Arterial blood was collected from untreated, ether-anesthetized rats into syringes containing sodium citrate (0.38% final concentration). Approximately 50 ml of blood were collected for each perfusion. This was kept at 37°C in the perfusion apparatus, and a gentle stream of humidifed 95% O_2/5% CO_2 was passed over the blood to maintain pH. Lungs were ventilated as described above. The perfusate flow was 8 ml/min, and a single-pass system was used. The lungs were pre-perfused with a Krebs-bicarbonate buffer containing 4% BSA for 30 minutes to clear the vasculature. After this stabilization period, the lungs were perfused for 4 minutes with blood. Platelet number, hematocrit and pH of the inflow and effluent blood perfusate were measured for each perfusion. Values for each of these were not different in the effluent blood perfusate of lungs from treated and control rats.

A sample of the blood was taken from the reservoir just prior to the perfusion, and samples of the effluent were collected every minute after switching to blood perfusate. Blood was collected into polypropylene tubes containing indomethacin (final concentration, 100 μM), and these were immediately spun in a centrifuge to obtain plasma. The plasma was then frozen at -70°C until analysis of prostanoids by radioimmunoassay. The concentration of TxB_2 was determined in unextracted plasma, and the concentration of 6-Keto $PGF_{1\alpha}$ was determined in plasma extracted as described below. In addition, for lungs from rats treated 14 days earlier with MCTP, samples of buffer from the last minute of the pre-perfusion period were collected as described above for determination of prostanoids.

Occasionally, platelet activation occurred during the process of drawing blood for the perfusion. This was indicated by a relatively high concentration of TxB_2 in the blood prior to perfusion. Therefore, any lung for which the preperfusion concentration of TxB_2 was greater than 0.2 ng/ml plasma was omitted from the study.

XIII. Determination of Prostanoids by Radioimmunoassay

A. General Procedure

The concentrations of thromboxane B_2 (TxB_2) and 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$), which are stable metabolites of thromboxane A_2 and prostacyclin (PGI_2), respectively, were determined in biological fluids by radioimmunoassay (RIA). Specific antibodies and antigens were purchased from Seragen Inc. (Boston, MA), and radioactive antigens (^3H) were purchased from Amersham (Arlington Heights, IL). The cross-reactivites of the antibodies as reported by Seragen, Inc., are shown in Table 1.

TABLE 1

Cross Reactivities (at 50% B/Bo) of Specific Antisera Used in Radioimmunoassays

	A	
	Anti-6-Keto $PGF_{1\alpha}$	Anti-TxB_2
B		
TxB_2	< 0.1	100
6-Keto-$PGF_{1\alpha}$	100	< 0.1
$PGF_{1\alpha}$	7.8	< 0.1
6-Keto-PGE_1	6.8	NR
$PGF_{2\alpha}$	2.2	< 0.1
PGE_1	0.7	< 0.1
PGE_2	0.6	< 0.1
PGD_2	< 0.1	< 0.1
PGA_1	< 0.1	< 0.1
PGA_2	< 0.1	< 0.1
PGB_1	< 0.1	< 0.1
PGB_2	< 0.1	< 0.1
15-Keto-$PGF_{2\alpha}$	< 0.1	NR
15-Keto-PGE_2	< 0.1	NR
Dihydroxy keto E_2	< 0.1	NR
Dihydroxy Keto $F_{2\alpha}$	< 0.1	NR
Dihydroxy Keto E_1	NR	< 0.1
Dihydroxy Keto $F_{1\alpha}$	NR	< 0.1
5-HETE	NR	< 0.1
12-HETE	NR	< 0.1
15-HETE	NR	< 0.1

As reported by Seragen, Inc.

Cross reactivity = $\frac{\text{amount A bound}}{\text{amount B bound}} \times 100$

HETE = hydroxyeicosatetraenoic acid

NR = not reported.

The antibodies were reconstituted in phosphate buffered-saline containing 0.5% gelatin (Difco Laboratories, Detroit, MI) (PBSG) at a dilution that would bind 40% of the labelled antigen ("trace") in the absence of unlabelled antigen. The trace was also prepared in PBSG. A stock standard solution of unlabelled antigen was prepared in PBSG, and this was diluted with the appropriate medium for working standards used in the assay. The appropriate medium for standards used in an RIA was the same biological fluid as the samples to be analyzed. Charcoal-stripped plasma, prepared as described below, was used to construct standard curves for determination of prostanoid concentration in plasma.

Equal volumes (100 µl each) of trace, antibody, and standard or sample were mixed in an assay tube. A complete standard curve was run with each assay. In addition, tubes were prepared for determination of non-specific binding of the trace to the antibody and for determination of total radioacivity bound in the absence of unlabeled antigen (B_o). The tubes were refrigerated for 12-24 hours, then decolorizing carbon (Norit^A, J.T. Baker Chemical Co., Phillipsburg, NJ) coated with dextran (T70, Pharmacia Fine Chemicals, Uppsala, Sweden) (dextran-coated charcoal) was used to separate the trace that was bound to antibody from the unbound trace. After exposure to the charcoal solution for 12 minutes, the tubes were spun in a centrifuge at 0°C (300 x g, 12 minutes). The supernatant, containing the antibody bound to the trace and to the unlabelled antigen, was decanted into scintillation vials and was mixed with 15 ml of scintillation cocktail (Safety Solve, Research Products International Corp., Mount Prospect, IL). The vials were placed in a liquid scintillation counter (Beckman LS-3150P) and were counted for 5 minutes for determination of radioactivity.

Non-specific binding was subtracted from all standards and samples, and the ratio of the amount of ^3H bound to the total possible ^3H bound (B_o) was

calculated. Standard curves were constructed as the logit of this ratio vs. the log of the amount of unlabelled antigen in the tube. The amount of antigen in the sample was then determined from this standard curve.

B. Preparation of charcoal-stripped plasma

Charcoal-stripped plasma was used as the medium in which standards were made for determination of prostanoids in plasma. Rats were treated with indomethacin (10 mg/kg, i.p.) one hour before blood was drawn from the abdominal aorta. Blood was collected into syringes containing sodium citrate (0.38% final concentration), and was spun for 10 minutes (600 x g). The plasma was then transferred to a clean tube, and a concentrated solution of dextran-coated charcoal was added (100 µl charcoal solution/1 ml plasma). This was vortexed, kept on ice for 15 min, then spun in a centrifuge at 0°C (300 x g, 12 min). The supernatant was then decanted and frozen until use.

C. Extraction of prostanoids

In some experiments, prostanoids were extracted with ethyl acetate before analysis by RIA (Jaffe and Behrman, 1974). Acidified samples were vortexed with ethyl acetate (3 x volume of sample), then were spun in a centrifuge (600 x g, 10 min). The supernatant was transferred to a clean tube and evaporated to dryness under nitrogen in an ice bath. The residue was dissolved in PBSG, and frozen until analysis by RIA.

To determine extraction efficiency, a minimal amount of radioactive antigen was added to a sample of the biological fluid before extraction. The radioactivity in the sample before and after extraction was determined. The extraction efficiency was the radioactivity in the extracted sample as a percentage of the radioactivity in the sample before extraction. Values for the concentrations of prostanoid in all extracted samples were corrected for extraction efficiency.

XIV. Drug Treatments

A. Ketanserin

Ketanserin was supplied as a gift from Janssen Pharmaceutica, Beerse, Belgium. Ketanserin (2.5 mg/kg) or its vehicle, distilled water, was administered by gavage twice daily. Treatment started three days after administration of MCTP and continued through day 14.

The effectiveness of the dosing regimen was verified in two studies. In the first study, the end-point examined was the change in the transmittance of light (shape change) observed in PRP in response to 5HT (Drummond and Gordon, 1975; Laubscher and Pletscher, 1979). At various times following administration of ketanserin, rats were anesthetized with ether, and blood was collected from the abdominal aorta for preparation of PRP by the first method described above. The shape change of the platelets in response to 5HT (creatinine sulfate complex, 1 μg), measured as the maximal decrease in light transmittance (Born, 1970), was observed using a Payton dual channel platelet aggregometer. In the second study, the effect of co-treatment with ketanserin on the vascular response to 5HT was examined in isolated, perfused lungs of MCTP-treated rats as described above.

B. Ibuprofen

Ibuprofen was supplied as a gift from The Upjohn Company (Kalamazoo, MI) by Mr. Peter Chelune. Sodium ibuprofenate (10 or 17.5 mg/kg ibuprofen) or its vehicle, saline, was administered to rats three times daily by gavage. Treatment began at the time of administration of MCTP and continued through the end of the study.

Drug effectiveness was determined as inhibition of the platelet aggregation response to sodium arachidonate, and as a decrease in the concentration of thromboxane B_2 in the plasma of treated rats.

C. Dazmegrel

UK38485 (Dazmegrel, 3-(1H-imidazol-1-yl-methyl)-2-methyl-1H-indole-1-propanoic acid) was a gift from Pfizer Central Research (Sandwich, Kent, England). Dazmegrel (50 mg/kg; dissolved in alkaline saline, pH=8.5) or its vehicle was administered twice daily by gavage starting at the time of administration of MCTP and continuing through the end of the study.

Drug effectiveness was determined as the decrease in the concentration of thromboxane B_2 in the plasma, platelet-rich plasma, or platelet-poor plasma of treated rats.

D. L-640,035

L-640,035 ((3-hydroxymethyl)dibenzo[b,f]thiepin-5,5-dioxide) was supplied as a gift from Merck Frosst Canada, Inc. Polyethylene glycol (approximate molecular weight = 200, diluted with an equal volume of distilled water) was used as the vehicle. L-640,035 (50 mg/kg) or its vehicle was administered by gavage three times daily starting on the day of administration of MCTP and continuing through the end of the study.

The effectiveness of this dosing regimen was confirmed as a decrease in the right ventricular pressor response to intravenous administration of a stable endoperoxide analogue and thromboxane mimic, U46619 (5z,9_α,11_α,13e,15s)-11,9-(epoxymethano)prosta-5,13-dien-1-oic acid (supplied by Mr. Peter Chelune of The Upjohn Company, Kalamazoo, MI). U46619, dissolved in ethanol and diluted with saline, was introduced through a catheter (PE10) positioned in the left femoral vein. Right ventricular pressure was measured as described above.

XV. Statistical Analysis

Data are expressed as mean $\pm$ S.E.M. In experiments having only two groups, the Student's t-test was used to compare means (Steel and Torrie, 1980).

The t-test for percentages was used to compare the survival of MCTP-treated and control rats. A completely random design one-way analysis of variance (ANOVA) was used to make comparisons among three or more groups. Prostanoid release from isolated, perfused lungs was analyzed using a mixed-design ANOVA, and individual comparisons were made with the least significance difference (lsd) test for between groups comparisons and Dunnett's test for within group comparisons. The effect of drug treatments on MCTP toxicity was evaluated using a two-way factorial ANOVA, and Tukey's ω-procedure was used to make individual comparisons. Homogeneity of variance was tested using the Fmax procedure, and, when data violated the assumption of homogeneity, logarithmic transformation of the data was performed. If the data remained non-homogeneous, pre-planned comparisons were made using the Wilcoxon-Mann-Whitney two-sample test (rank sum test). In all cases, a 95% confidence level was used as the criterion for significance.

RESULTS

I. Dose/Response Relation for MCTP

A single intravenous injection of 5 mg MCTP/kg body weight produces pulmonary injury and pulmonary hypertension in rats, and by 14 days after treatment, right ventricular hypertrophy has developed (Bruner et al., 1983). However, using this dose of MCTP, in several studies we had experienced high mortality (approximately 50%). The objective of this study was to determine a dose of MCTP which would produce right ventricular enlargement (RVE) by day 14 after treatment but would result in fewer deaths among the treated rats.

On day 0, rats received an intravenous injection of DMF or one of 4 doses of MCTP. The doses used were 2, 3, 4, and 5 mg/kg. The concentration of MCTP in DMF was adjusted so that each dose was delivered in a volume of 0.5 ml DMF/kg, and controls received an equivalent volume of DMF. Fourteen days later, the rats were killed and lung injury and RVE were assessed.

By day 14, 4 of the 9 rats treated with the highest dose of MCTP (5 mg/kg) had died (mortality = 44%). No other animals in this study died over the 14 day period (n=7 for all other groups). Table 2 shows the effect of the different doses of MCTP on body weight. Body weight gain was suppressed by MCTP treatment at doses greater than 2 mg/kg.

RVE developed by day 14 at each dose of MCTP tested (Figure 5A). Wet lung weight/body weight was also elevated in all groups of MCTP-treated rats (Figure 5B), as were lavage protein concentration and LDH activity (Figures 5C and 5D).

TABLE 2

Effect of Treatment with Various Doses of MCTP on Body Weight Gain

Treatment[a]	BW_o	BW_F	Δ BW
DMF	261± 6	338± 9	+77± 8
2 mg MCTP/kg	256± 7	330±10	+74± 5
3 mg MCTP/kg	259± 6	279±19[b]	+20±19[b]
4 mg MCTP/kg	262± 6	270±18[b]	+9±21[b]
5 mg MCTP/kg	261±13	217±16[b]	-45± 9[b]

[a]Rats received either i.v. MCTP or DMF on day 0 and were killed 14 days later. BW_o = initial body weight; BW_F = final body weight. N = 5-7.

[b]Significantly different from DMF.

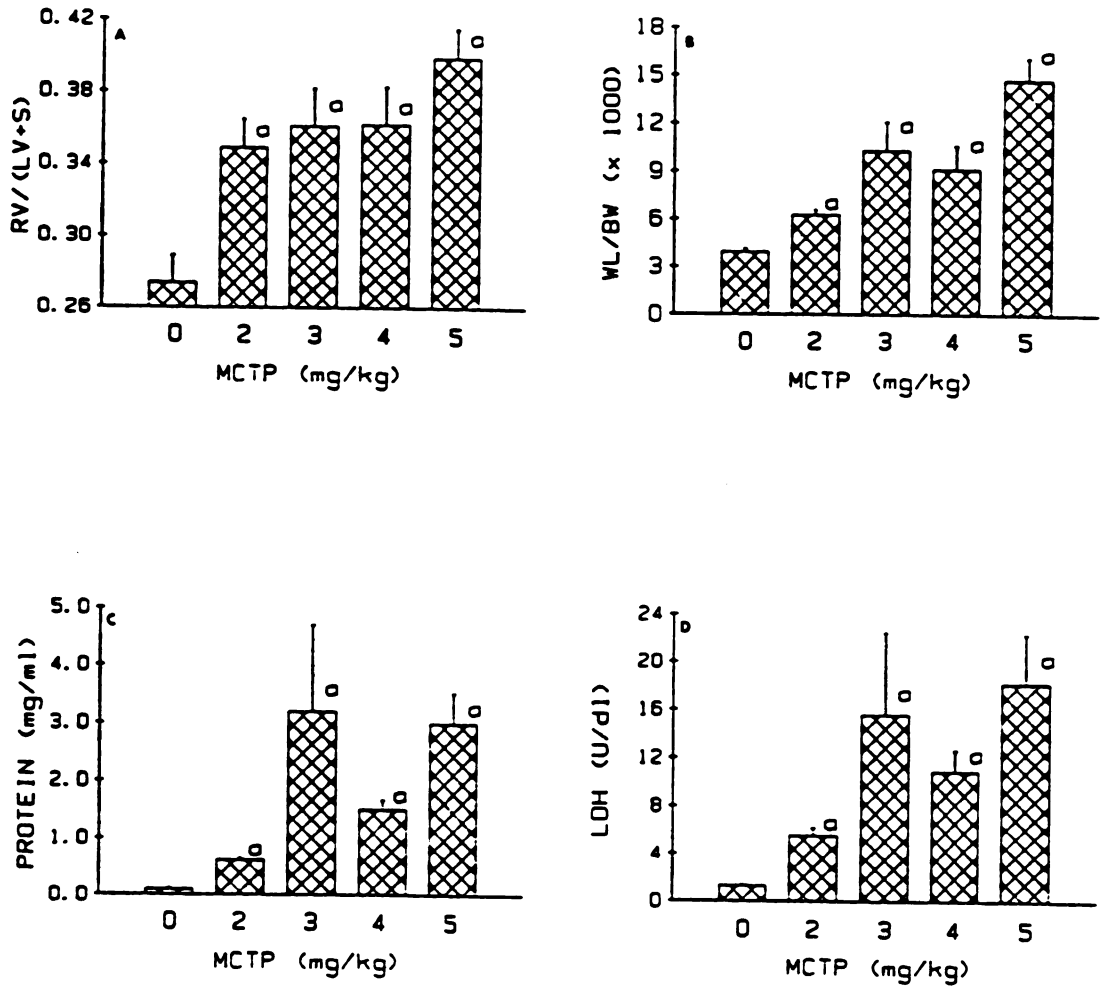

Figure 5. Dose/response relation for MCTP. Rats received either i.v. MCTP or DMF on day 0 and were killed on day 14. (A) RV/(LV+S) = right ventricular enlargement; (B) WL/BW: WL = wet lung weight; BW = body weight; (C) Lavage fluid protein concentration; (D) Lavage fluid LDH activity. N = 5-7. a = significantly different from control (0 mg/kg = DMF).

On the basis of these results, doses of 3.5, 4, and 5 mg MCTP/kg were chosen for subsequent studies.

II. Histopathology (Development of MCTP-induced Toxicity)

The purpose of this study was to correlate the biochemical and physiological alterations caused by MCTP with changes identified histologically. To this end, rats received either MCTP (3.5 mg/kg), DMF, or saline intravenously on Day 0, and were killed 3, 5, 8, or 14 days later. One group of animals was used for the determination of lung injury (lavage protein concentration, LDH activity, and lung weight) and vascular leak, while in a second group pulmonary arterial pressure was measured and the lungs were subsequently fixed and processed for histological examination. All groups of animals were used for the determination of RVE. Animals from all groups were killed on the same day.

Lungs were fixed via the vasculature and the airways as described in Methods. Tissues were examined by light microscopy by Dr. James Reindel without prior knowledge of treatment group. Alterations were graded on a 0 (no change) to ++++ (most severe change) scale. There were no differences at any time observed histologically in the lungs from rats which received saline and DMF, therefore only DMF data are presented.

Three days after treatment with MCTP, lavage LDH activity was elevated, vascular leak was evident, and the wet/dry lung weight ratio was decreased (Table 3). Lavage protein concentration and the wet lung/body weight ratio were not affected by treatment at this time, and PAP and RV/(LV+S) were not different from control. Changes at the light microscope level were minimal in rats treated 3 days earlier with MCTP (Table 4). There was a mild accumulation of non-proteinaceous edema fluid within the perivascular and peribronchiolar connective tissue which was most pronounced around large- and medium-sized blood vessels

TABLE 3

Development of Toxicity Due to MCTP

Days After Treatment	Treatment[a]	WL/BW (x1000)	WL/DL	Lavage Protein (mg/ml)	Lavage LDH Activity U/dl)	$\frac{^{125}\text{I-Lung}}{^{125}\text{I-Blood}}$	PAP (mmHg)	RV/(LV+S)
3	DMF	4.0±0.1	5.6±0.2	0.12±0.01	1.8±0.2	17.6± 0.9	19.8±0.7	0.274±0.008
	MCTP	4.1±0.1	5.3±0.1[b]	0.16±0.02	4.7±1.1[b]	25.8± 2.7[b]	19.1±1.2	0.263±0.006
5	DMF	3.7±0.2	5.3±0.2	0.14±0.01	2.3±0.3	20.1± 2.5	19.4±2.0	0.273±0.008
	MCTP	5.4±0.4[b]	6.2±0.2[b]	0.54±0.12[b]	17.3±3.0[b]	74.7±34.7[b]	21.1±1.0	0.275±0.007
8	DMF	3.6±0.1	5.6±0.5	0.10±0.01	1.4±0.1	21.4± 3.5	17.4±2.1	0.258±0.010
	MCTP	5.5±0.5[b]	5.9±0.4	1.02±0.29[b]	17.9±3.8[b]	56.9± 9.6[b]	23.7±3.7	0.283±0.007
14	DMF	3.7±0.1	5.1±0.3	0.14±0.02	2.0±0.1	20.0± 0.5	20.1±1.1	0.266±0.008
	MCTP	11.5±1.3[b]	7.3±0.5[b]	3.01±0.48[b]	11.4±1.6[b]	75.9± 6.9[b]	41.2±5.2[b]	0.364±0.012[b]

[a]On day 0 rats received either MCTP (3.5 mg/kg) or DMF i.v. WL = wet lung weight; BW = final body weight; DL = dry lung weight; PAP = mean pulmonary arterial pressure. N = 5-8.

[b]Significantly different from DMF on the same day.

TABLE 4

Histopathologic Changes Following Treatment with MCTP

Days After Treatment	Treatment[a]	Edema			Cell Infiltrations			WBC Margination
		Perivascular	Alveolar Wall	Alveolar Lumen	Perivascular	Alveolar Wall	Alveolar Lumen	
3	DMF	0	0	0	0/+	0	0	0
	MCTP	+	0	0	+	0/+	0	+/++
5	DMF	0	0	0	+	0	0	0
	MCTP	+++	+	0	+	+	+	
8	DMF	0	0	0	0/+	0	0	0
	MCTP	++	++	++	++	++	++	++
14	DMF	+	0	0	0/+	0	0	0/+
	MCTP	+++	++	+++	+++	+++	+++	+

[a]Rats received either MCTP (3.5 mg/kg) or DMF on day 0. Lungs were fixed and processed as described in Methods. Tissues were examined blind and severity was graded from 0 (no change) to ++++ (severe change). Values represent means of all tissues evaluated. WBC = white blood cell. N = 5-9.

and bronchioles. Lymphatics in this interstitial space were distended with edema fluid. A mild perivascular cellular infiltrate was observed, consisting largely of lymphomononuclear cells with fewer polymorphonuclear cells (PMNs). Less frequently, and in more severely affected tissue, an increased margination of white blood cells (WBCs) was noted in muscular and partially muscular vessels, large foamy macrophages were noted in alveolar spaces, and slightly hypertrophic Type II pneumocytes were observed in alveoli.

By day 5 after treatment, wet lung weight, wet/dry lung weight ratio, and lavage LDH activity and protein concentration were elevated in treated rats (Table 3). Vascular leak was also evident although pulmonary hypertension and RVE were not manifest at this time. Perivascular and peribronchiolar edema was more pronounced than at day 3 (Table 4), and was associated with smaller-sized vessels and airways. Lymphatics in the interstitial space were dilated. Perivascular cell infiltrates were also more prominent than at day 3, again consisting primarily of large lymphomononuclear cells, some of which were hypertrophic, and occasional mast cells. Marginated WBCs were frequently seen. In patchy areas, alveolar septal walls were thickened with edema fluid. In addition, there was a mild cellular infiltrate in the septal wall, mainly lympomononuclear cells. Hypertrophied alveolar Type II cells were observed, and enlarged macrophages were noted in the alveolar walls and lumen. In areas of marked perivascular edema, separation of vascular smooth muscle cells and hypertrophy of individual cells were observed.

Lavage protein concentration, lavage LDH activity, wet lung weight, and vascular leak were increased at day 8, but RVE was not evident (Table 3). Two of the five rats for which PAP measurements were obtained had pressures in the normal range so that the mean of all treated animals at this time was not significantly different from control. However, the PAPs of the other 3 treated

rats were greater than the PAP of any control rat. When the histopathology of these 5 treated rats was compared, one treated rat with normal PAP showed only mild changes, not very different from control lungs. Changes in the other rat with normal PAP were not unlike changes in treated rats with elevated PAP. At day 8, variability in the severity and extent of lesions observed histologically was noted among the eight treated rats evaluated, and also among and within the lung lobes of each rat. Perivascular edema was associated with large, medium, and small muscular vessels, and perivascular cellular infiltrates were mostly lymphomononuclear cells with fewer PMNs (usually eosinophils). WBC margination was prominent and platelet thrombi were also occasionally observed in vessels of various sizes. Accumulation of edema fluid in the alveolar septae and alveolar lumen was more pronounced at day 8 than at earlier times examined (Table 4). Hypercellularity of the alveolar wall was observed, due to infiltration of lymphomononuclear cells and hypertrophy of interstitial cells. Large foamy macrophages, containing phagocytized cellular debris, were commonly seen in the alveolar spaces. In the bronchiolar epithelium, surface blebbing of Clara cells was less pronounced. A general proliferative response was evident: hypertrophy of interstitial cells and alveolar Type II cells, and increased numbers of mitotic figures in the perivascular and alveolar septal wall interstitium, alveolar sacs, and bronchiole epithelium were observed. Nuclear dust from degenerate cells was frequently associated with mitotic figures. In areas of severe perivascular edema and marked cellularity endothelial cell hypertrophy and separation of medial smooth muscle cells were observed, as well as hypertrophic smooth muscle cell nuclei.

By day 14, wet lung weight, the wet/dry lung weight ratio, lavage protein concentration, lavage LDH activity, and vascular leak were increased in MCTP-treated rats (Table 3). Pulmonary hypertension and RVE were also evident.

Alterations observed by light microscope were similar to those seen at day 8, only increased in severity and extent (Table 4). In addition, marked changes were noted in the bronchiolar epithelium, and the bronchiolar epithelium had extended and lined alveolar ducts and sacs, replacing the normally thinner epithelium. Alveolar lumens often contained fibrin strands and macrophages with phagocytized cellular debris. Although vascular alterations were, for the most part, mild, endothelial cell blebbing and smooth muscle cell separation and hypertrophy were frequent observations. Although morphometric analysis was not performed, in areas of severe injury some vessel walls appeared slightly thickened.

In summary, the first alterations observed, at day 3 after MCTP treatment, were vascular leak and perivascular edema. By day 5 lavage protein concentration was elevated, and infiltration of lymphomononuclear cells was observed in perivascular connective tissue. Lung lesions progressed as indices of lung injury worsened. By day 8 there was marked cell proliferation in the airways, and edema fluid and increased numbers of macrophage were noted in the alveoli. These alterations were more extensive and severe at day 14, and there was some indication of smooth muscle hypertrophy in pulmonary vessels at this time. For the most part, vascular alterations were unimpressive, and the most marked alterations were observed in the airways.

III. Diet Restriction and MCTP-induced Cardiopulmonary Toxicity

Hayashi and coworkers (1979) demonstrated that reduction of food intake that was associated with retarded growth inhibited the cardiopulmonary changes induced by MCT and prolonged survival in MCT-treated rats. Because diet and nutritional status can alter metabolism of xenobiotic agents (Kato and Gillette, 1965; Mgbodile et al., 1973), it was unknown whether this protective effect was due to decreased bioactivation of MCT to MCTP. The purpose of this study was

to determine whether diet restriction had a similar protective effect against the cardiopulmonary response to MCTP, which apparently does not require bioactivation to exert its toxicity (Bruner et al., 1986).

A 2x2 factorial design was used. Rats received either MCTP (5 mg/kg, i.v.) or DMF, and were either allowed to eat ad libitum or were restricted to 9 g food/rat/day. All rats were killed 14 days after treatment.

A. Effect of dietary restriction on MCTP-induced cardiopulmonary toxicity

Body weights for the 4 groups of animals are shown in Figure 6. MCTP-treated rats that were allowed free access to food gained less weight than control rats which ate ad lib. The body weights of MCTP-treated and control rats restricted to 9 g food/rat/day declined at a similar rate. Only two animals died during the course of the study; both were MCTP-treated, diet-restricted rats, and both died on day 12 after administration of MCTP.

Diet-restricted animals in both the MCTP-treated and control groups had lower liver weights than the respective ad lib groups (Table 5). Kidney weight and SGOT were not affected by MCTP treatment or by diet restriction. BUN was elevated in DMF diet-restricted rats compared to the ad lib controls, perhaps reflecting protein catabolism in diet-restricted rats.

MCTP treatment produced an increase in lavage protein concentration (Figure 7A) which was significantly lower in MCTP-treated, diet-restricted rats than in MCTP-treated rats which ate ad lib. The LDH activity was elevated in the lavage fluid of MCTP-treated rats from both the diet-restricted and ad lib groups, and there was no significant difference between these two groups (Figure 7B). The lung/body weight ratio was elevated by MCTP treatment and diet restriction attenuated this increase (Figure 7C).

MCTP-treated rats allowed to eat ad lib had RVE, as indicated by an increased RV/(LV+S) ratio (Figure 7D). However, in MCTP-treated rats fed a

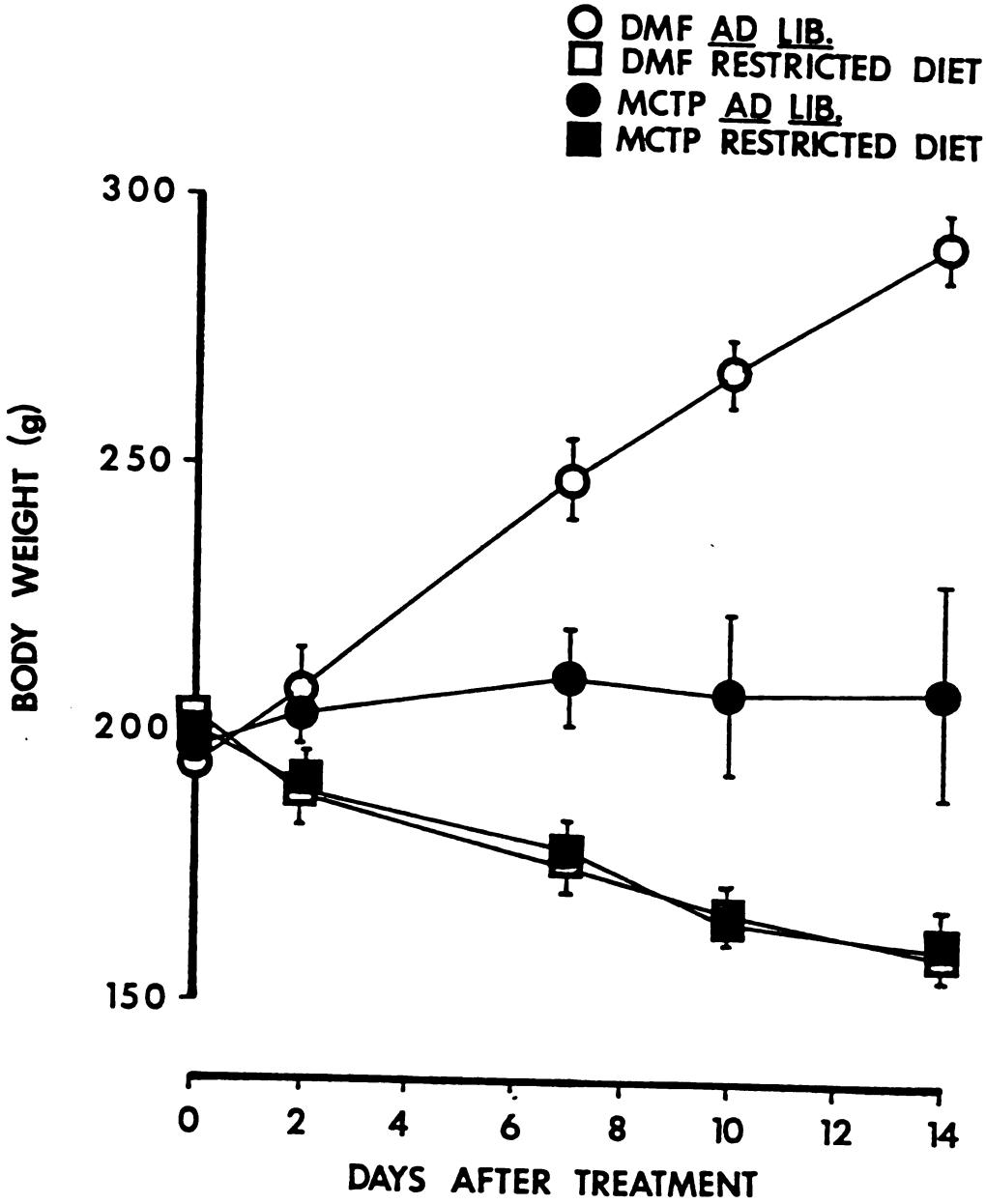

Figure 6. Effect of diet restriction on body weight of MCTP-treated rats. Rats were treated with either MCTP (5 mg/kg) or DMF and were allowed to eat ad libitum or were restricted to 9 g food/rat/day. Open circles = DMF ad libitum; open squares = DMF-restricted diet; closed circles = MCTP ad libitum; closed squares = MCT-restricted diet. N = 7-10.

TABLE 5

Liver Weight, Kidney Weight, Blood Urea Nitrogen (BUN) and Serum Glutamatic Oxalacetic Transaminase (SGOT) Activity in Diet-restricted, MCTP-treated Rats

	Treatment[a]			
	DMF		MCTP	
	Ad Lib	Restricted Diet	Ad Lib	Restricted Diet
Liver Wt/BW (x100)	4.19±0.12	3.38±0.10[b]	3.98±0.13	3.43±0.11[c]
Kidney Wt/BW (x100)	8.0±0.2	9.4±0.3	9.2±0.8	9.6±0.2
BUN (mg %)	17± 2	29± 4[b]	22±3	30± 4
SGOT (SF Units/ml)	101±10	123±20	91±7	99±11

[a]Rats were treated with MCTP (5 mg/kg) or DMF and were allowed to eat ad libitum or were restricted to 9 g food/rat/day. Animals were killed 14 days after treatment. BUN = blood urea nitrogen; SGOT = serum glutamic oxalacetic transaminase. N = 7-10.

[b]Significantly different from DMF ad libitum group.

[c]Significantly different from MCTP ad libitum group.

Figure 7. Effect of diet restriction on (A) lavage protein concentration, (B) lavage LDH activity, (C) lung weight/body weight, and (D) RV/(LV+S) in MCTP-treated rats. Rats were treated with a single i.v. injection of either MCTP (5 mg/kg) or DMF and were allowed to eat ad libitum or were restricted to 9 g food/rat/day. Animals were killed 14 days after treatment. Open bars = ad libitum; solid bars = restricted diet. N = 7-10. a = significantly different from DMF ad libitum group. b = significantly different from DMF-restricted diet group. c = significantly different from MCTP ad libitum group.

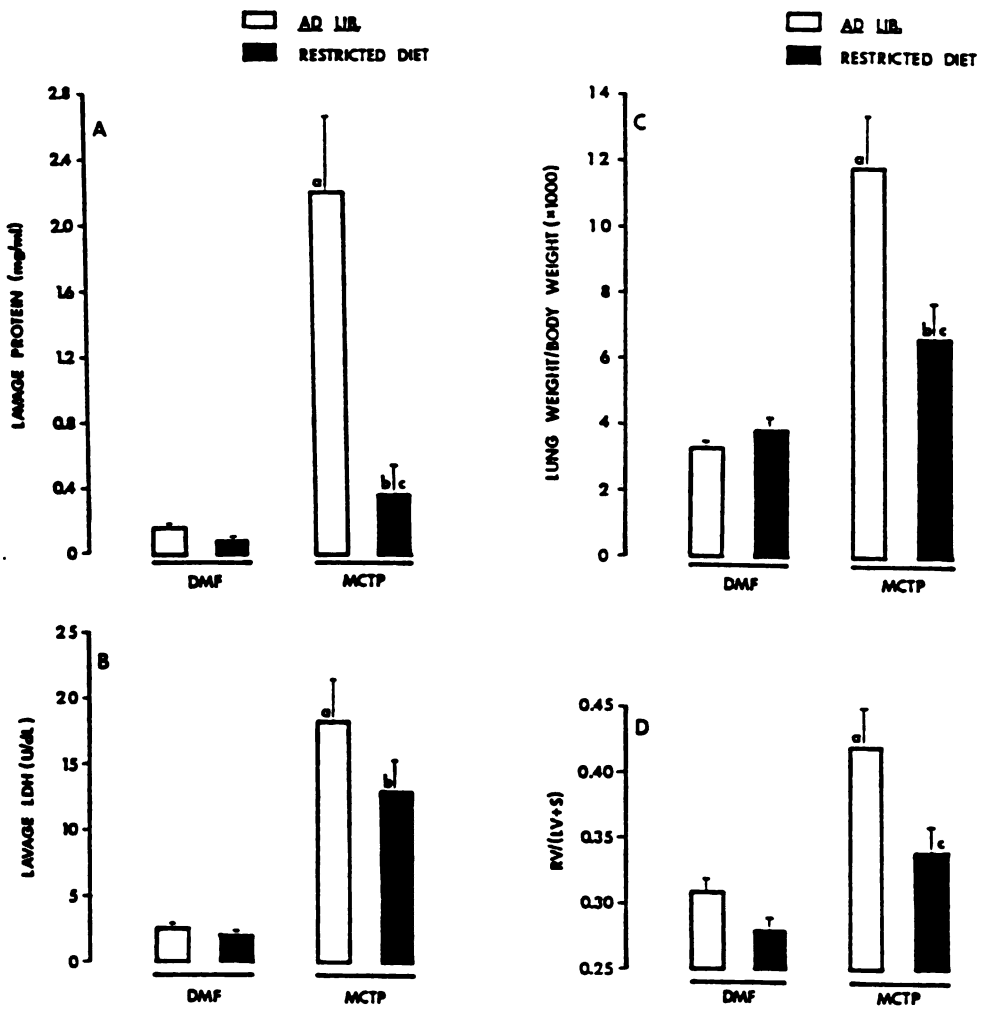
AD LIB.
RESTRICTED DIET
A
LAVAGE PROTEIN (mg/ml)
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
a
b|c
DMF
MCTP
C
LUNG WEIGHT/BODY WEIGHT (×1000)
14
12
10
8
6
4
2
0
a
b|c
DMF
MCTP
B
LAVAGE LDH (U/dL)
25
20
15
10
5
0
a
b
DMF
MCTP
D
RV/(LV+S)
0.45
0.40
0.35
0.30
0.25
a
c
DMF
MCTP

Figure 7

restricted diet, this ratio was significantly less than in MCTP-treated rats fed ad lib and was not different from diet-restricted controls.

B. Effect of Diet Restriction on Survival of MCTP-treated Rats

Because it appeared that diet restriction afforded some protection from the cardiopulmonary toxicity of MCTP, it was of interest to determine whether a reduction in food intake allowed MCTP-treated animals to live longer. As shown in Figure 8, between 10 and 28 days following treatment with MCTP, the fraction of rats surviving was greater in the food-restricted group. However, after day 29, there was no significant difference in the number of rats surviving, suggesting that the effect of diet restriction to prolong survival time of MCTP-treated rats is temporary.

Animals surviving through day 41 were killed, and lavage fluid LDH activity and RVE were determined. The lavage fluid LDH activity was not significantly different between the two groups (Table 6) and was close to that of rats not treated with MCTP (compare Figure 7B). The RV/(LV+S) was significantly lower in the diet-restricted group, suggesting that the effect of diet restriction against MCTP-induced RVE is sustained through 40 days following treatment.

The results of these studies indicate that reduction of food intake or body weight gain attenuates the cardiopulmonary effects of MCTP. Therefore it is important in drug treatment studies to choose a dose of the drug which does not by itself retard weight gain.

IV. Effect of Thrombocytopenia on MCTP-induced Pulmonary Hypertension

One of the first experiments performed to test whether the platelet might be involved in the response to MCTP was to examine the toxicity of MCTP in rats with decreased numbers of circulating platelets (Hilliker et al., 1984a). In

Figure 8. Effect of diet restriction on the number of MCTP-treated rats surviving. Rats were treated with MCTP (5 mg/kg) and were allowed free access to food (solid line) or were restricted to 9 g food/rat/day (broken line). Initial number of rats/group = 15. Asterisks indicate significant differences from ad libitum group on the same day.

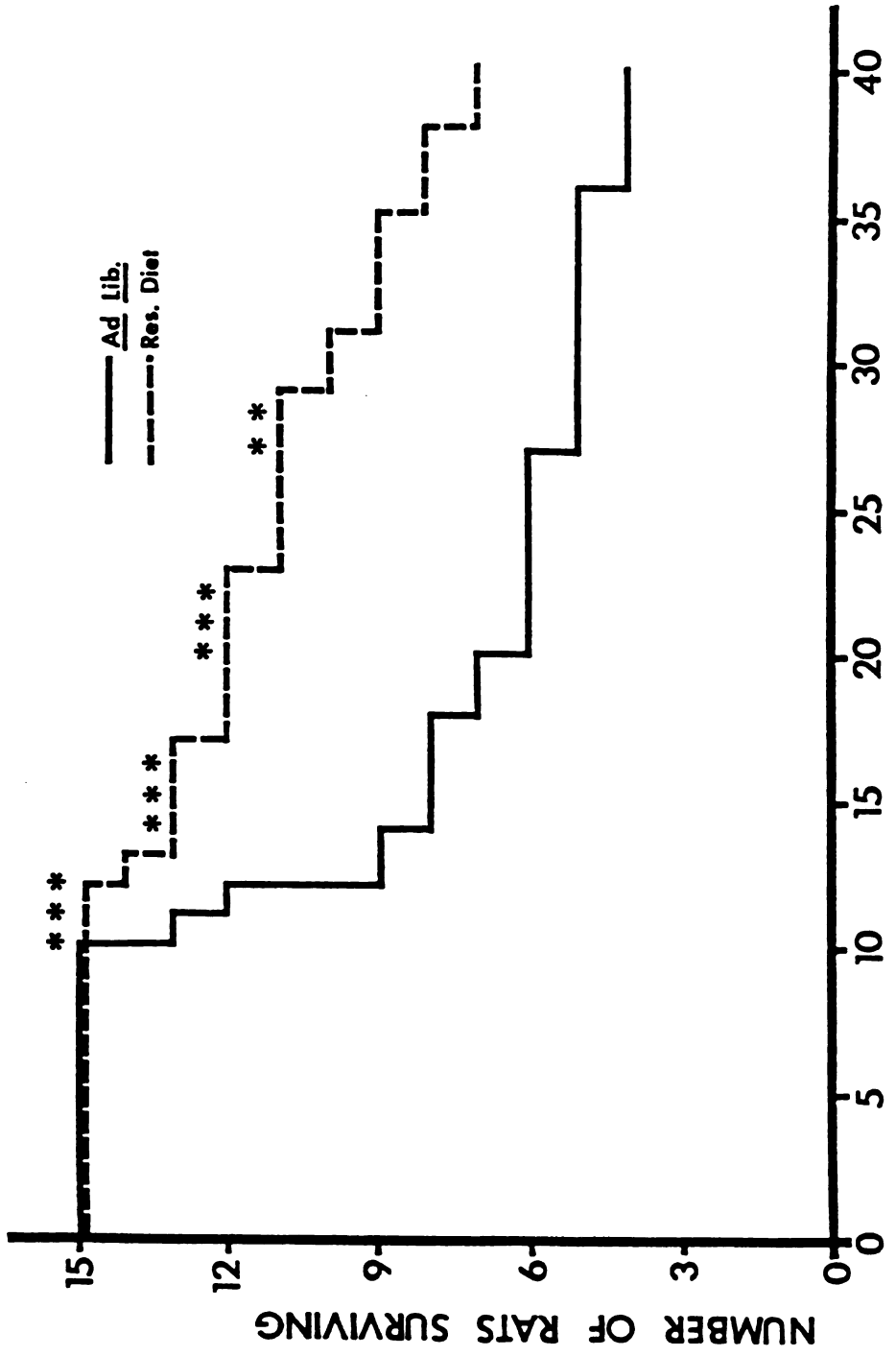
Ad Lib.
Res. Diet
***
****
***
**
NUMBER OF RATS SURVIVING
0
3
6
9
12
15
0
5
10
15
20
25
30
35
40
DAYS SURVIVING

Figure 8

TABLE 6

Lavage LDH Activity and Right Ventricular Enlargement in Rats Surviving 41 Days After MCTP Treatment

	Treatment[a]	
	Ad Lib	Restricted Diet[b]
Lavage LDH (U/dl)	4.5±0.8	4.9±0.7
RV/(LV+S)	0.434±0.063	0.294±0.020[c]
N	4	7

[a]Rats were treated with i.v. MCTP (5 mg/kg). Animals surviving 41 days were killed and their lungs were lavaged as described in METHODS.

[b]Restricted diet = 9 g food/rat/day.

[c]Significantly different from ad libitum group.

preliminary experiments a goat anti-rat platelet antiserum (PAS) was administered to rats, and the number of circulating platelets could be decreased to 10-20% of control for a period of 2 days. Treatment with the PAS for a longer period of time caused the animals to become ill as evidenced by ruffled appearance, inactivity and loss of weight, and some of the animals died. When rats treated with MCTP on day 0 were made thrombocytopenic with this PAS from days 0-2, lung injury and RVE assessed at day 14 were not different from MCTP-treated rats with a normal platelet count (Hilliker et al., 1984a). However, when rats were made thrombocytopenic from days 3-5 or days 6-8, RVE was not as severe at day 14 as in rats treated with control serum (CS). This protective effect was greater when the period of thrombocytopenia was from days 6-8 than days 3-5. MCTP-induced lung injury was not altered in rats killed at day 14 by co-treatment with the PAS. This attenuation of RVE suggested that the platelet may be involved in MCTP-induced pulmonary hypertension since RVE is thought to be a consequence of the sustained increase in pulmonary arterial pressure in this model. To determine whether decreasing the number of circulating platelets attenuated the increase in pulmonary arterial pressure as well as RVE, MCTP-treated rats were co-treated with PAS, and pulmonary artery pressure was measured directly.

It was first necessary to develop another antiserum to rat platelets, and this was performed in a goat as described in METHODS. The potency and specificity of the PAS was determined in vivo. The PAS was then used to examine the effect of severe thrombocytopenia ($< 5\%$) and moderate thrombocytopenia (10-25%) on MCTP-induced cardiopulmonary toxicity.

A. Antiserum Characterization

Preliminary experiments were performed in untreated rats to determine a non-toxic dose of the PAS which would deplete platelets. Treatment with

up to 2.0 ml (i.p., a single dose) of the new PAS produced no overt signs of toxicity such as loss of weight or ruffled appearance, and platelet number was depressed by approximately 95%. In a more extensive study, rats were bled from the tail for an initial platelet count, and they were then treated with 1.5 ml (i.p.) of CS or PAS at time 0. These rats received a "booster" of 0.5 ml (i.p.) CS or PAS (respectively) at 36 hours. Blood samples were also taken from the tail at various times between 6 hours and 6 days after treatment with CS or PAS, and platelet count and hematocrit were determined. Total white blood cell counts and differential white cell counts were determined at 12 hours.

There was no difference in body weight of rats treated with CS or PAS through 6 days after treatment (Table 7). The fact that these rats did not gain weight over this 6-day period could be due to treatment with the sera or to the trauma of being bled so frequently.

Platelet number was decreased by 6 hours in rats treated with PAS relative to rats receiving the CS (Figure 9). Platelet number remained low through 48 hours, and it had returned to normal by 144 hours after treatment. Using this dosing regimen, platelet number in PAS-treated rats was 5-10% of the control value (which was approximately $10^6/\mu l$). Hematocrit tended to decrease (non-significant) over time after treatment in both groups (Figure 10), probably due to frequent blood sampling from these rats. At 96 hours after treatment, hematocrit was significantly lower in PAS-treated rats than controls.

Total white blood cell number was not different in rats treated with PAS and CS 12 hours earlier (Table 8). The distribution of white cells was largely unaffected by treatment with PAS, except for a greater percentage of basophils in PAS-treated rats.

TABLE 7

Effect of Treatment with PAS on
Body Weight (g)

Time After Treatment (Days)	Treatment[a]	
	CS	PAS
0	359±8	368± 8
1	346±7	339±10
2	338±6	333±10
3	342±6	334±10
4	341±5	332±10
6	343±8	337±10

[a]Rats received CS or PAS at time 0 (1.5 ml) and again 36 hours later (0.5 ml). N = 4-5.

Figure 9. Platelet number in the blood of rats treated with CS or PAS. Rats received i.p. CS or PAS at time 0 (1.5 ml) and again at 36 hours (0.5 ml). Blood was taken from the tail for determination of platelet number. N = 4-5. * = significantly different from CS at the same time.

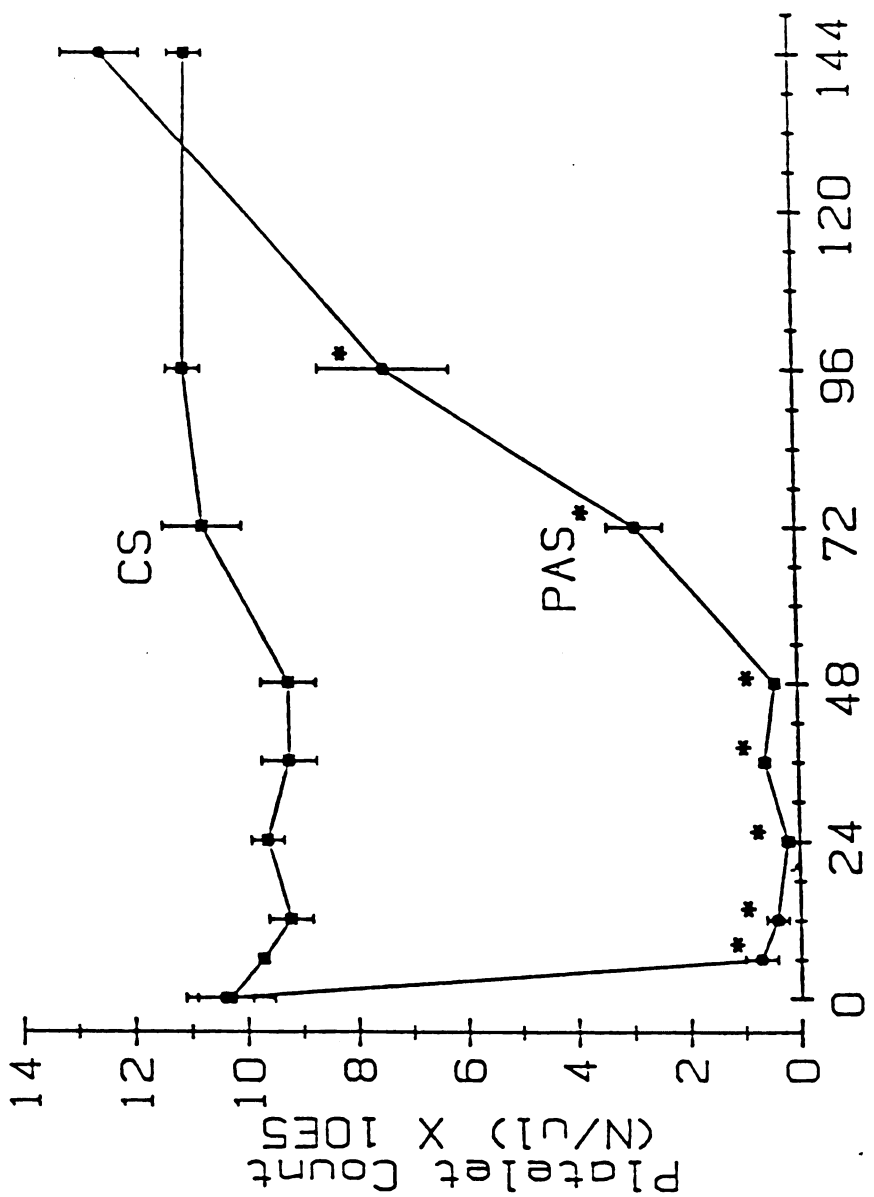
Platelet Count
(N/cl) X 10E5
14
12
10
8
6
4
2
0
0
24
48
72
96
120
144
Time (hours)
CS
PAS
*

Figure 9

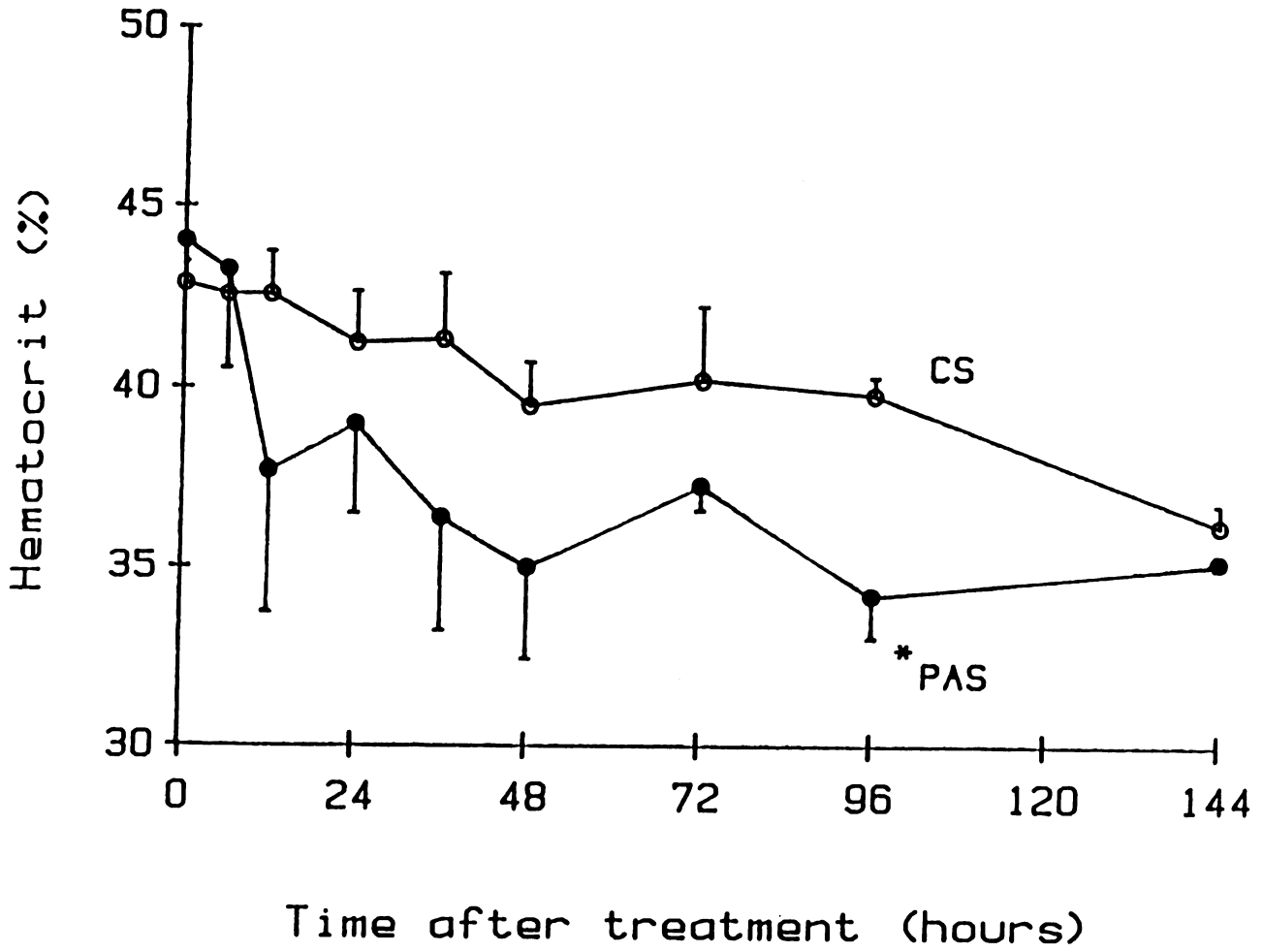

Figure 10. Hematocrit of the blood of rats treated with CS or PAS. Rats were treated as described in the legend to Figure 9. N = 4-5. * = significantly different from CS at the same time.

TABLE 8

White Blood Cell Count in the Blood of Rats
Treated with CS or PAS

Cell Type	Treatment[a]	
	CS	PAS
Total (white blood cells/μl)	10633±1733	13043±1501
Lymphocytes (%)	58.8±5.4	56.3±2.6
Neutrophils (%)	37.8±5.3	39.7±2.6
Monocytes (%)	2.7±0.9	2.1±0.7
Eosinophils (%)	0.2±0.2	0.3±0.2
Basophils (%)	0.2±0.2	1.6±0.2[b]

[a]Rats were treated at time 0 with CS or PAS (1.5 ml), and blood samples were taken from the tail 12 hours later. Cells were counted as described in METHODS. N = 4-5.

[b]Significantly different from CS control.

B. Effect of Severe Thrombocytopenia

Using the dosing regimen described above for PAS, the circulating platelet number was decreased to approximately 5% of normal. This was referred to as "severe" thrombocytopenia, because in later experiments a lower dose of PAS was used to decrease platelet number to 10-25% of normal. Severe thrombocytopenia was induced in MCTP-treated rats because it was hypothesized that a greater degree of thrombocytopenia might afford greater protection from the cardiopulmonary toxicity of MCTP. In addition, it was of interest to determine whether thrombocytopenia altered the early lung injury or development of pulmonary hypertension. This was tested by examining MCTP-induced toxicity at day 8 in rats co-treated with PAS.

In the first experiment, rats were treated with DMF or MCTP, and were co-treated with CS or PAS from days 6-8 as described above. It was also considered that, because in the original experiment greater protection had been afforded when the period of thrombocytopenia was later in the development of toxicity (i.e., days 6-8 compared to days 3-5) (Hilliker et al., 1984a), waiting still longer to treat with PAS may attenuate the response to a greater degree. To test this, MCTP-treated rats were co-treated with the PAS from days 8-10 or from days 10-12. In these latter experiments, all rats were treated with MCTP or DMF on day 0 and were killed on day 14.

1. Rats made severely thrombocytopenic from days 6-8

This study was undertaken for two purposes. The first was to confirm the effect of thrombocytopenia on MCTP-induced RVE at day 14 and to extend that observation to direct measurement of pulmonary arterial pressure. The second purpose was to assess the effect of decreasing platelet number on the development of pulmonary hypertension and lung injury. To this end, rats were treated with MCTP (3.5 mg/kg, i.v.) on day 0, and were co-treated with CS or PAS

(i.p.) on days 6 and 7. Rats were killed 8 or 14 days later, and lung injury and pulmonary hypertension were assessed.

a. Effect on MCTP toxicity at day 8. Body weight gain was unaffected at day 8 by MCTP treatment or by co-treatment with PAS (Table 9). The wet lung weight, wet/dry lung weight ratio, and lavage LDH activity and protein concentration were elevated in MCTP-treated rats, and these indices of injury were not affected by co-treatment with PAS. RVE was not evident at this time. The normal platelet count for a rat is approximately $10x10^5$ pt/µl and, using this value, platelet number in the PAS-treated rats was less than 10% of normal. Platelet number was lower in MCTP-treated rats co-treated with PAS than in DMF controls.

Pulmonary arterial pressure was significantly higher in MCTP-treated rats co-treated with CS at this time (Figure 11). Pulmonary artery pressure in thrombocytopenic MCTP-treated rats was slightly higher than controls, but it was not significantly different from DMF/PAS rats or from MCTP/CS rats. Right ventricular pressure tended to be higher in MCTP/CS rats than in DMF/CS rats, but this difference did not reach statistical significance.

b. Effect on MCTP toxicity at day 14. Treatment with MCTP resulted in elevation of wet lung weight, lavage LDH activity and lavage protein concentration by day 14 (Table 10). These indices of lung injury were not different in MCTP-treated rats co-treated with CS or PAS. The wet/dry lung weight ratio was not altered at this time by treatment with MCTP or PAS. In this group of rats, treatment with PAS reduced the number of circulating platelets to less than 5% of normal ($10x10^5$/µl). There was no difference in platelet number in the blood of DMF/PAS and MCTP/PAS rats.

Pulmonary artery pressure was elevated by MCTP treatment at day 14, and this increase was not attenuated in rats with reduced platelet

TABLE 9

Effect of Severe Thrombocytopenia (Days 6-8) on Toxicity
8 Days After Treatment with MCTP

	Treatment[a]			
	DMF		MCTP	
	CS	PAS	CS	PAS
$BW_{initial}$ (g)	216±6	217± 6	217± 7	220±4
BW_{final} (g)	295±6	277±10	267±10	272±9
WL/BW (x1000)	4.1±0.1	4.2±0.1	7.1±0.5[b]	6.1±0.3[c]
WL/DL	4.9±0.1	5.0±0.1	6.1±0.1[b]	5.8±0.2[c]
Lavage LDH Activity (U/dl)	2.2±0.2	2.6±0.2	26.3±4.1[b]	26.8±1.9[c]
Lavage Protein (mg/ml)	0.10±0.02	0.13±0.03	2.64±0.51[b]	1.40±0.24[c]
RV/(LV+S)	0.267±0.011	0.268±0.010	0.287±0.014	0.303±0.007
Platelet number ($x10^5/\mu l$)	ND	0.78±0.14	ND	0.31±0.05[c]

[a]Rats were treated on day 0 with MCTP (3.5 mg/kg) or DMF and on days 6 and 7 with CS or PAS. Rats were killed on day 8. BW = body weight; WL = wet lung weight; DL = dry lung weight; Platelet number was determined 24 hours after the first treatment with PAS. ND = not determined. N = 5-6.

[b]Significantly different from DMF/CS.

[c]Significantly different from DMF/PAS.

Figure 11. Effect of severe thrombocytopenia on (A) mean pulmonary artery pressure (PAP) and (B) right ventricular pressure (RVP) 8 days after treatment with MCTP (3.5 mg/kg) or DMF. On day 0 rats received i.v. MCTP or DMF, and then were co-treated with i.p. CS or PAS on days 6 and 7. PAP and RVP were measured on day 8. N = 5-6. a = significantly different from DMF/CS.

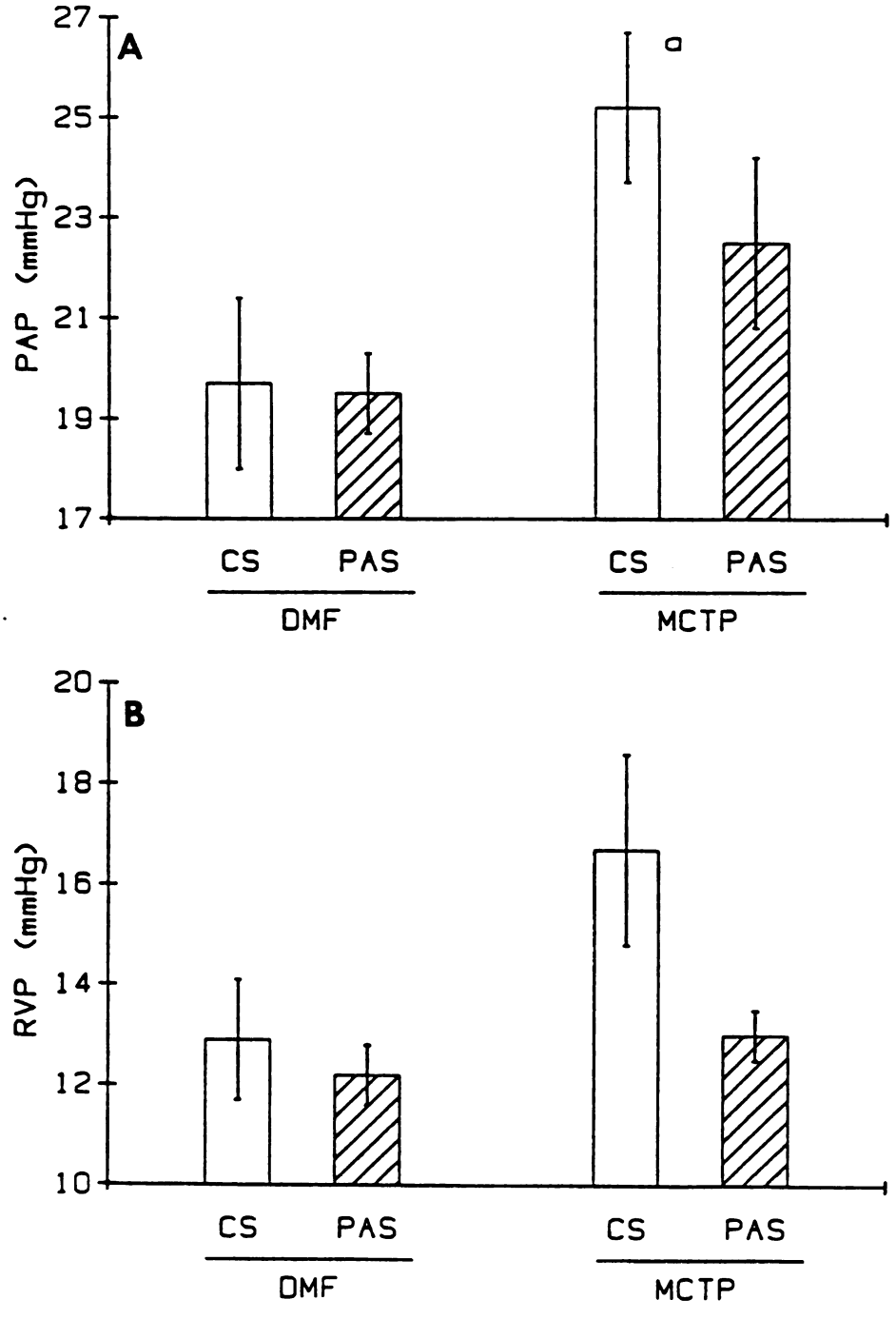
A
a
PAP (mmHg)
27
25
23
21
19
17
CS
PAS
CS
PAS
DMF
MCTP
B
RVP (mmHg)
20
18
16
14
12
10
CS
PAS
CS
PAS
DMF
MCTP

Figure 11

TABLE 10

Effect of Severe Thrombocytopenia (Days 6-8) on Toxicity
14 Days after Treatment with MCTP

	Treatment[a]			
	DMF		MCTP	
	CS	PAS	CS	PAS
$BW_{initial}$ (g)	210±4	213±4	210±4	215±4
BW_{final} (g)	318±6	315±9	304±8	291±7
WL/BW (x1000)	4.0±0.1	4.1±0.1	6.7±0.4[b]	7.5±0.5[c]
WL/DL	5.3±0.1	5.3±0.1	6.0±0.2	6.2±0.3
Lavage LDH Activity (μ/dl)	2.8±0.2	2.9±0.3	6.5±1.2[b]	8.1±1.6[c]
Lavage Protein (mg/ml)	0.08±0.02	0.17±0.08	1.12±0.29[b]	1.31±0.34[c]
Platelet Number ($x10^5/\mu l$)	ND	0.48±0.14	ND	0.37±0.07

[a]Rats were treated as described in Table 9, and were killed on day 14. BW = body weight; WL = wet lung weight; DL = dry lung weight; Platelet number was determined 24 hours after the first treatment with PAS. ND = not determined. N = 6-11.

[b]Significantly different from DMF/CS.

[c]Significantly different from DMF/PAS.

numbers (Figure 12). The same was true of right ventricular pressure. MCTP-treated rats had developed RVE by this time, and this was not different in MCTP-treated rats co-treated with CS or PAS (Figure 13).

The results of these studies indicated that this degree of thrombocytopenia neither protected against lung injury nor attenuated the pulmo nary hypertensive response to MCTP. Although both at day 8 and at day 14 there was a slight decrease in pulmonary artery pressure in MCTP-treated rats made thrombocytopenic relative to MCTP-treated rats receiving the control serum, these differences did not reach statistical significance. This was also true of RVE at day 14.

2. Rats made thrombocytopenic from days 8-10 or 10-12

In a previous study, less severe RVE had been observed when the period of thrombocytopenia was from days 6-8 than when it was from days 3-5 (Hilliker et al., 1984a). This observation raised the possibility that when platelets were depleted later in the development of MCTP toxicity, more protection was afforded. To test this, rats were treated with MCTP (3.5 mg/kg) or DMF on day 0, and were co-treated with CS or PAS either at days 8 and 9 or at days 10 and 11 to induce thrombocytopenia. Rats were killed at day 14, and lung weight and right ventricular enlargement were assessed.

In rats treated with PAS from days 8-10, the platelet number was less than 5% of the platelet number in rats treated with CS (Table 11). However, co-treatment with PAS did not attenuate the MCTP-induced increase in wet lung weight or RVE.

When the window of thrombocytopenia was moved to days 10-12, the increase in lung weight and RVE caused by MCTP were not altered by co-treatment with PAS (Table 12). Although platelet number was less than 5% of

Figure 12. Effect of severe thrombocytopenia on (A) mean pulmonary artery pressure (PAP) and (B) right ventricular pressure (RVP) 14 days after treatment with MCTP (3.5 mg/kg) or DMF. Rats were treated as described in Figure 11 and PAP and RVP were measured on day 14. N = 6-11. a = significantly different from DMF/CS. b = significantly different from DMF/PAS.

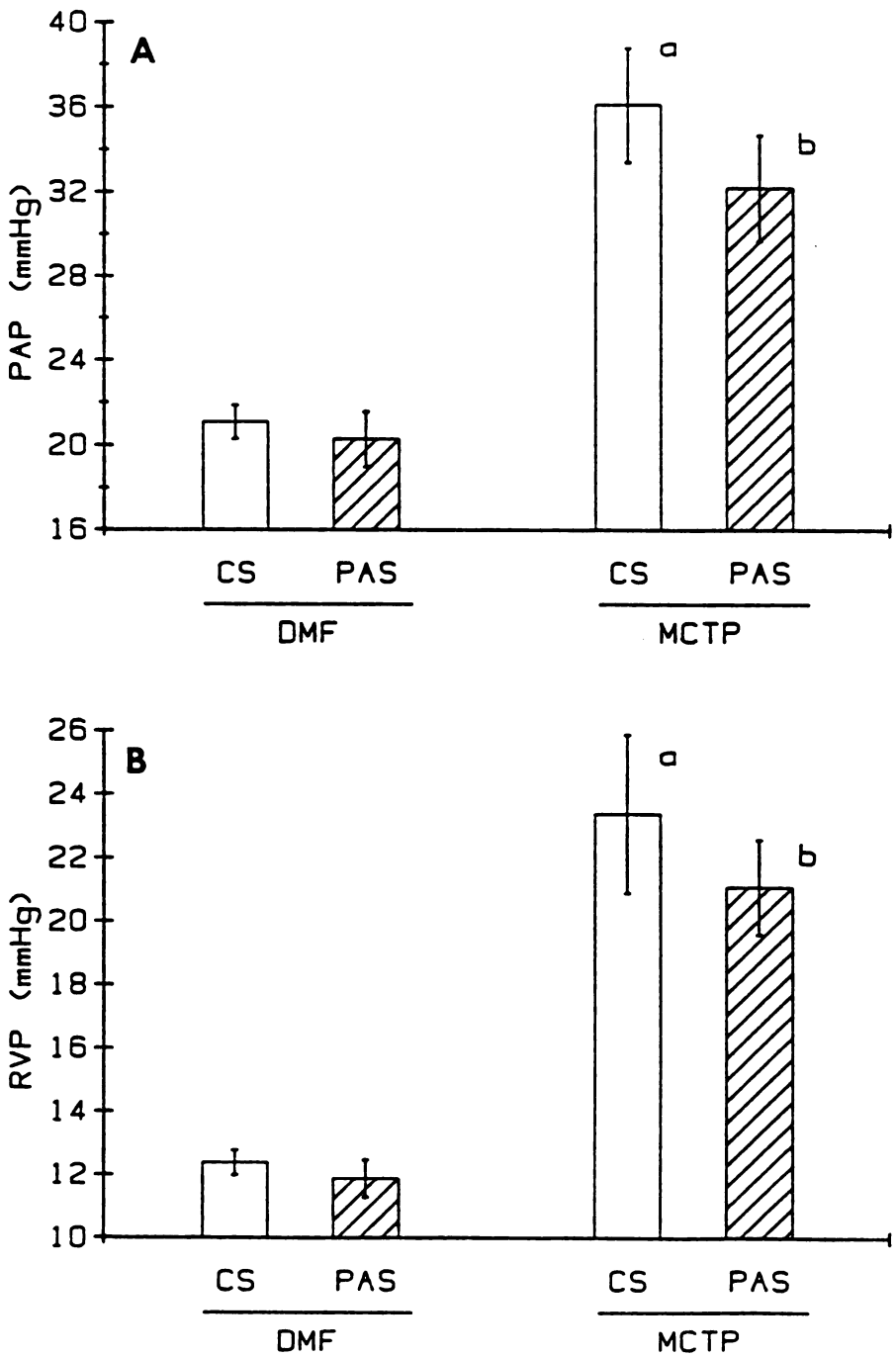
A
PAP (mmHg)
40
36
32
28
24
20
16
a
b
CS
PAS
CS
PAS
DMF
MCTP
B
RVP (mmHg)
26
24
22
20
18
16
14
12
10
a
b
CS
PAS
CS
PAS
DMF
MCTP

Figure 12

Figure 13. Right ventricular enlargement at day 14 in MCTP-treated rats co-treated with PAS. Rats were treated as described in Figure 11. N = 6-11. a = significantly different from DMF/CS. b = significantly different from DMF/PAS.

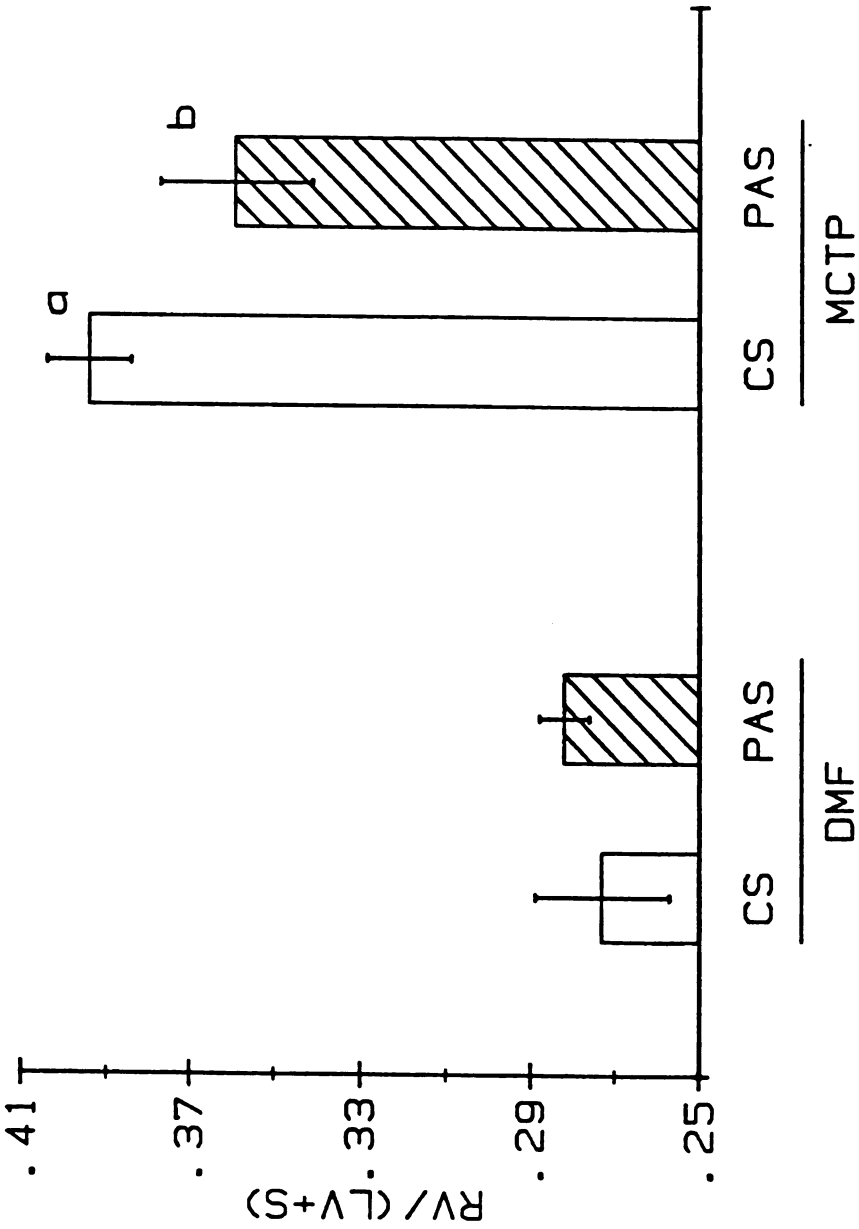

Figure 13

TABLE 11

Effect of Severe Thrombocytopenia (Days 8-10) on the Toxicity of MCTP

	Treatment[a]			
	CS	PAS	CS	PAS
Platelet number ($x10^5/\mu l$)	11.0±0.2	0.24±0.1[b]	11.4±0.3	0.13±0.1[d]
WL/BW (x1000)	3.8±0.1	4.0±0.1	6.7±0.4[b]	8.2±1.2[c]
RV/(LV+S)	0.280±0.011	0.258±0.013	0.415±0.052[b]	0.420±0.028[c]

[a]Rats received MCTP (3.5 mg/kg) or DMF on day 0 and were then co-treated with either CS or PAS on days 8 and 9. Lung weight and right ventricular enlargement were assessed on day 14. Platelet number was determined 24 hours after the first dose of CS or PAS. WL = wet lung weight; BW = body weight. N = 3-4.

[b]Significantly different from DMF/CS.

[c]Significantly different from DMF/PAS.

[d]Significantly different from MCTP/PAS.

TABLE 12

Effect of Severe Thrombocytopenia (Days 10-12) on the Toxicity of MCTP

	Treatment[a]			
	CS	PAS	CS	PAS
Platelet number ($x10^5/\mu l$)	ND	2.1±2.1	ND	0.10±0.02
WL/BW (x1000)	3.7±0.1	3.8±0.1	7.0±0.5[b]	8.6±1.1[c]
RV/(LV+S)	0.263±0.007	0.281±0.006	0.373±0.018[b]	0.396±0.021[c]

[a]Rats were treated with MCTP (3.5 mg/kg) or DMF on day 0, and were then co-treated with either CS or PAS on days 10 and 11. Lung weight and right ventricular enlargement were assessed on day 14. Platelet number was determined 24 hours after the first dose of PAS. ND = not determined. WL = wet lung weight; BW = body weight. N = 3-5.

[b]Significantly different from DMF/CS.

[c]Significantly different from DMF/PAS.

normal in MCTP rats co-treated with PAS, DMF rats co-treated with PAS had a circulating platelet number approximately 20% of normal.

The results of these studies indicate that moving the window of thrombocytopenia to times later in the development of toxicity does not alter the response to MCTP.

C. Effect of Moderate Thrombocytopenia

The studies in which the circulating platelet number was depressed to less than 5% of normal failed to confirm the finding of a protective effect due to co-treatment with PAS. It was hypothesized that there may be a critical level for platelets, and that dropping the circulating platelet number below that level may of itself induce injury. Accordingly, a lower dose of the same PAS was given to rats to decrease platelet number to approximately 20% of normal. This was the degree of thrombocytopenia that occurred in the earlier study (Hilliker et al., 1984a) in which attenuated RVE was found. Rats were given MCTP (3.5 mg/kg) or DMF on day 0, and then were co-treated with CS or PAS on days 6 (0.75 ml, i.p.) and 7 (0.5 ml, i.p.). All rats were killed on day 14, and lung injury and pulmonary hypertension were assessed.

In a separate experiment using this same protocol, rats treated with PAS were bled from the tail daily through day 14, and platelet number was determined.

1. Effect on pulmonary hypertension

The platelet number in rats which received PAS was 23-24% of the platelet number in rats which received CS (Table 13). At this degree of thrombocytopenia, the increases in wet lung weight, lavage LDH activity and lavage protein concentration due to MCTP treatment at day 14 were not affected by co-treatment with PAS. Body weight gain was suppressed in MCTP-treated rats receiving the CS, but not in those receiving the PAS. The wet/dry lung

TABLE 13

Effect of Moderate Thrombocytopenia on MCTP-induced Toxicity

	Treatment[a]			
	DMF		MCTP	
	CS	PAS	CS	PAS
$BW_{initial}$ (g)	247±5	249±4	250±4	247±4
BW_{final} (g)	347±6	333±9	323±7[b]	323±5
WL/BW (x1000)	3.5±0.1	3.4±0.1	5.9±0.4[b]	5.0±0.2[c]
WL/DL	5.1±0.1	5.0±0.1	5.8±0.2[b]	5.4±0.1
Lavage LDH Activity (U/dl)	1.7±0.2	2.0±0.3	7.9±1.3[b]	6.3±0.7[c]
Lavage Protein (mg/ml)	0.083±0.004	0.100±0.009	1.39±0.28[b]	1.14±0.23[c]
Platelet Number ($10^5/\mu l$)	10.1±0.6	2.3±0.4[b]	9.7±0.6	2.3±0.3[d]

[a]Rats were given MCTP (3.5 mg/kg) or DMF on day 0. They were co-treated with CS or PAS from days 6-8, and were killed on day 14. WL = wet lung weight; BW = body weight; DL = dry lung weight; Platelet number was determined 24 hours after the first CS or PAS treatment. N = 7-14.

[b]Significantly different from DMF/CS.

[c]Significantly different from DMF/PAS.

[d]Significantly different from MCTP/CS.

weight ratio was also elevated only in MCTP-treated rats with normal platelet numbers.

RVE, present in MCTP-treated rats with a normal platelet count, was abolished in MCTP-treated rats with a decreased platelet number (Figure 14). Pulmonary hypertension developed in MCTP-treated rats co-treated with CS, but in MCTP-treated rats made moderately thrombocytopenic, pulmonary artery pressure was not different from control (Figure 15). This was true of the increase in right ventricular pressure was well.

These results indicated that at a relatively modest degree of thrombocytopenia, MCTP-treated rats do not develop pulmonary hypertension and RVE. Lung injury is not attenuated by decreasing the circulating platelet number when examined at day 14. This confirms the previous finding that thrombocytopenia affords a protective effect against RVE, and extends that finding to protection against elevation of pulmonary artery pressure.

2. Platelet rebound

The purpose of this experiment was to examine the effect of co-treatment with PAS on platelet number through day 14 following MCTP treatment. Rats were given i.v. MCTP (3.5 mg/kg) or DMF on day 0. On days 6 and 7 they received i.p. either CS or PAS. One group of DMF- and MCTP-treated rats co-treated with PAS ("bled") was bled from the tail immediately prior to the first injection of PAS and every 24 hours thereafter through day 14. Blood samples were taken from the tails of another group of DMF-and MCTP-treated rats co-treated with PAS ("not bled") only 24 hours after the first injection of PAS and again at day 14. Blood samples from rats co-treated with CS were only taken at day 14. Rats were killed on day 14, and lung injury and RVE were assessed.

As in previous studies, the increases in wet lung weight and lavage protein concentration due to MCTP were not affected at day 14 by

Figure 14. Effect of moderate thrombocytopenia on right ventricular enlargement in MCTP-treated rats. Rats were given i.v. MCTP (3.5 mg/kg) or DMF on day 0, and were co-treated with CS or PAS from days 6-8. RV/(LV+S) was assessed on day 14. N = 7-14. a = significantly different from DMF/CS. b = significantly different from MCTP/CS.

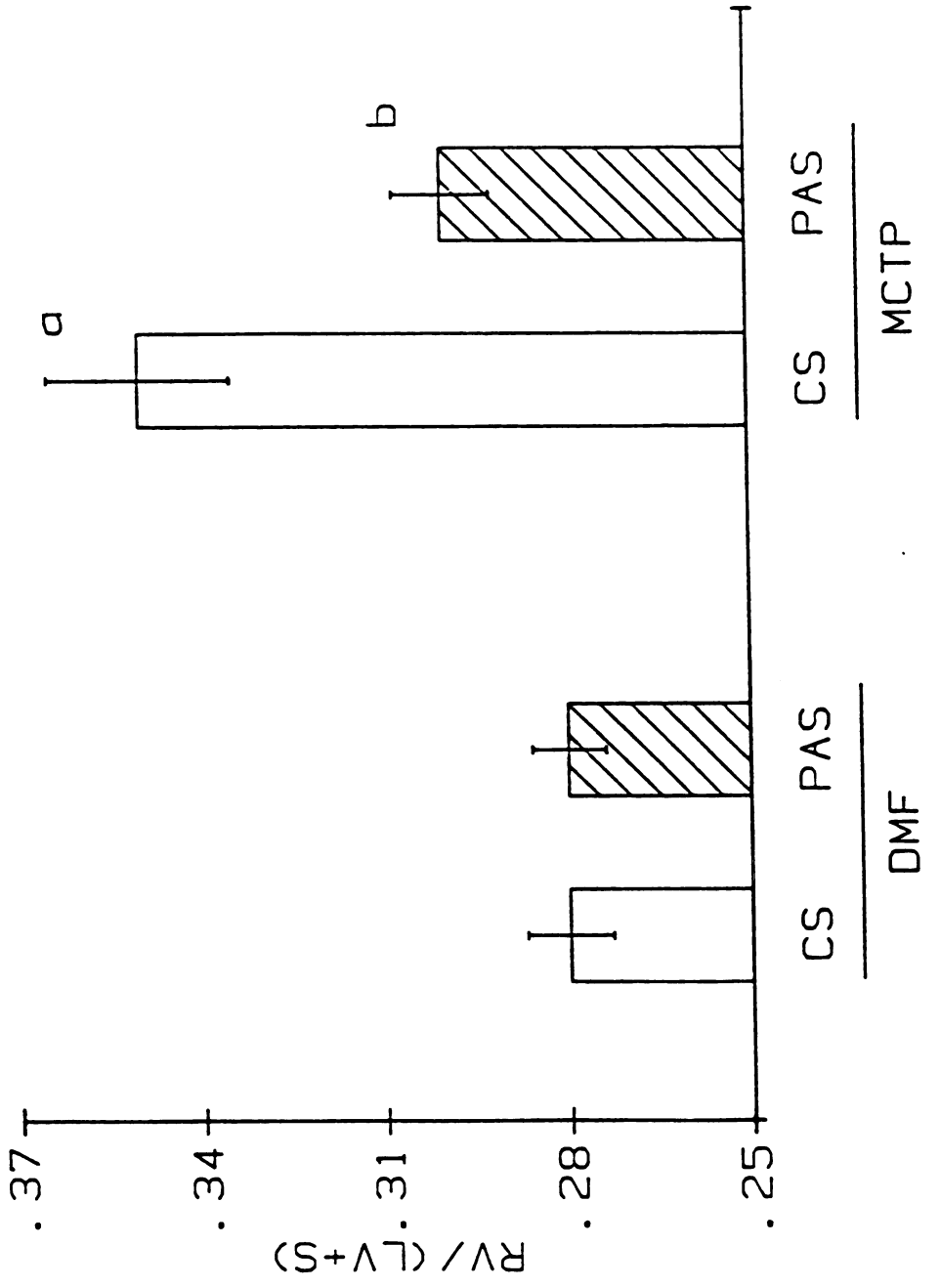

Figure 14

Figure 15. Effect of moderate thrombocytopenia on (A) mean pulmonary artery pressure (PAP) and (B) right ventricular pressure (RVP) in MCTP-treated rats. Rats were given i.v. MCTP (3.5 mg/kg) or DMF on day 0, and were co-treated with CS or PAS from days 6-8. PAP and RVP were measured on day 14. N = 7-14. a = significantly different from DMF/CS. b = significantly different from MCTP/CS.

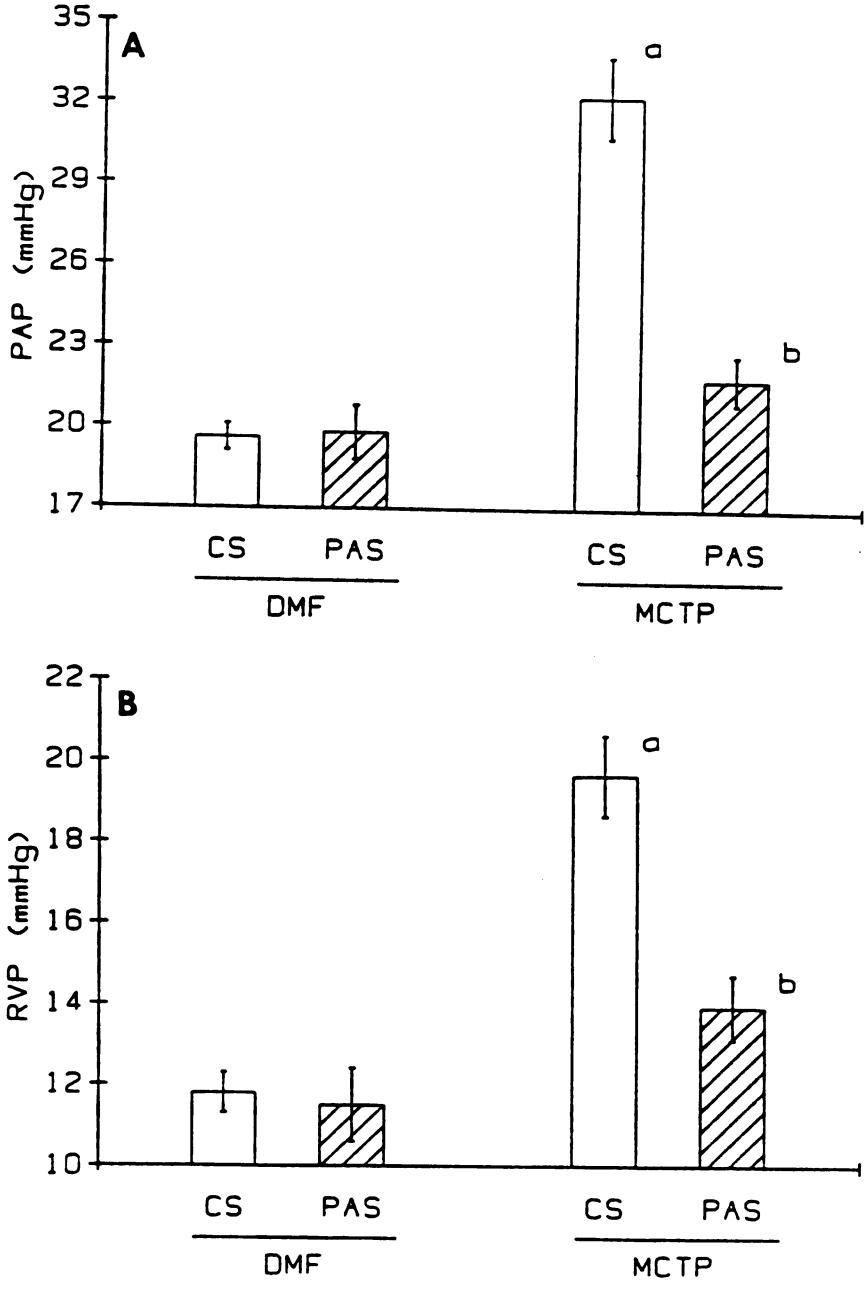
A
PAP (mmHg)
35
32
29
26
23
20
17
a
b
CS
PAS
DMF
CS
PAS
MCTP
B
RVP (mmHg)
22
20
18
16
14
12
10
a
b
CS
PAS
DMF
CS
PAS
MCTP

Figure 15

decreasing platelet number (Table 14). Lavage LDH activity was significantly higher in MCTP-treated rats co-treated with PAS than in those co-treated with CS. Body weight at day 14 was decreased in MCTP/PAS rats compared to DMF/PAS or MCTP/CS rats. RVE developed in MCTP-treated rats with a normal platelet number, but not in MCTP-treated rats made thrombocytopenic.

The platelet number in PAS-treated rats 24 hours after the first dose of PAS was approximately 12% of normal (Table 15). The platelet number in the bled group of rats prior to injection of PAS was slightly but significantly different in DMF ($11.6 \pm 0.4 \times 10^5/\mu l$) and MCTP-treated ($10.0 \pm 0.5 \times 10^5/\mu l$) rats ($p < 0.05$). Platelet number in the bled rats remained below 20% of the pre-PAS value through day 8 for both DMF- and MCTP-treated rats (Figure 16). On days 9 and 10 the platelet number was significantly higher in MCTP-treated rats, but there was no difference between these two groups at any other time. From day 11 through day 14, platelet number was above the pre-PAS value for both groups of rats, in MCTP-treated rats reaching approximately twice the normal number of circulating platelets (Figure 16). The magnitude of the overshoot tended to be greater in MCTP-treated rats than controls, but this difference was not statistically significant. On day 14 after MCTP (day 8 after PAS), platelet number was significantly higher in both DMF- and MCTP-treated rats co-treated with PAS relative to their respective CS controls (Table 15). There was no difference in platelet number at day 14 in the bled and not bled groups receiving either DMF or MCTP.

These results indicate that not only does treatment with PAS decrease platelet number during the window of thrombocytopenia, but it also causes an overshoot such that platelet numbers are greater than normal later in the progression of MCTP toxicity. This raises a question as to whether the protective effect of treatment with PAS on MCTP toxicity is due to the early

TABLE 14

Effect of Thrombocytopenia on Body Weight, Lung Injury, and Right Ventricular Enlargement in MCTP-treated Rats

	Treatment[a]			
	DMF		MCTP	
	CS	PAS	CS	PAS
$BW_{initial}$ (g)	220± 5	212±2	214± 5	209± 2
BW_{final} (g)	320±13	296±7	288±10	246±10[c,d]
WL/BW (x1000)	3.6±0.2	3.8±0.1	6.4±0.5[b]	8.3±1.6[c]
Lavage LDH Activity (U/dl)	2.2±0.1	2.2±0.1	9.9±2.2[b]	13.8±0.9[c,d]
Lavage Protein (mg/ml)	0.14±0.02	0.13±0.02	1.84±0.42[b]	2.28±0.71[c]
RV/(LV+S)	0.249±0.010	0.245±0.009	0.394±0.033[b]	0.313±0.014[d]

[a]Rats were treated on day 0 with MCTP (3.5 mg/kg) or DMF, were co-treated with CS or PAS on days 6 and 7, and were killed on day 14. BW = body weight; WL = wet lung weight.

[b]Significantly different from DMF/CS.

[c]Significantly different from DMF/PAS.

[d]Significantly different from MCTP/CS.

TABLE 15

Platelet Number in MCTP-treated Rats Receiving PAS

Days After Treatment with		Treatment[a]			
		DMF		MCTP	
MCTP	PAS	CS	PAS	CS	PAS
7	1	ND	1.2±0.1	ND	1.2±0.1
14	8	10.5±0.4	14.4±0.9[b]	10.7±0.7	16.7±1.0[c]

[a]Rats received MCTP (3.5 mg/kg) or DMF on day 0, and received either CS or PAS on days 6 and 7. Values are platelet number x $10^5/\mu l$. ND = not determined. N = 7.

[b]Significantly different from DMF/CS.

[c]Significantly different from MCTP/CS.

Figure 16. Effect of treatment with PAS on platelet number in MCTP-treated (solid line) and DMF (vehicle) (broken line) rats. Rats were treated with MCTP as described in Table 14, and received PAS at days 6 (0.75 ml) and 7 (0.5 ml). Blood was taken from the tail at 24 hour intervals for the determination of platelet number. Platelet number in the blood of each rat immediately prior to the first injection of PAS was used to calculate 100%. * = significantly different from vehicle on the same day.

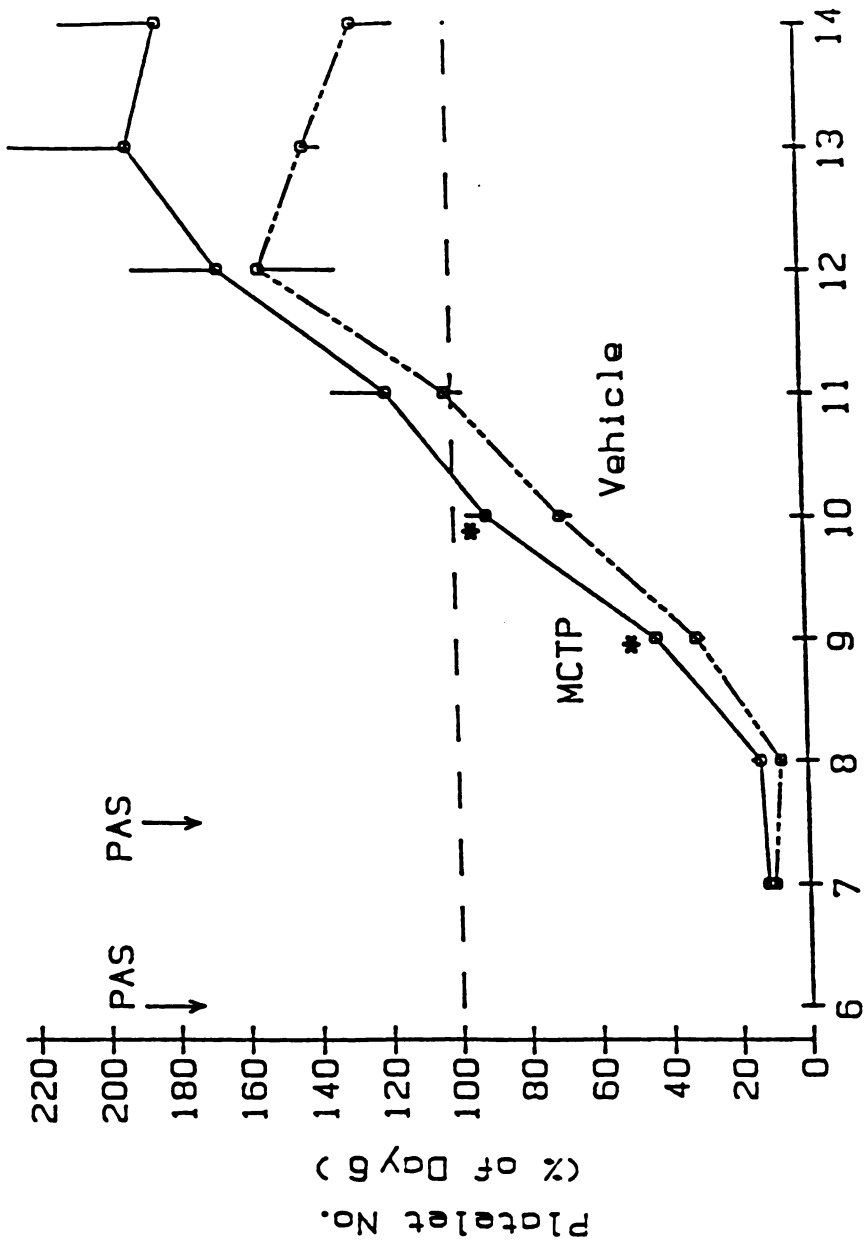
Platelet No.
(% of Day 6)
220
200
180
160
140
120
100
80
60
40
20
0
PAS
PAS
MCTP
Vehicle
*
*
6
7
8
9
10
11
12
13
14
Days after MCTP or Vehicle

Figure 16

event (decreased platelet numbers) or the later event (increased platelet numbers).

V. Examination of the Role of 5HT in MCTP-induced Cardiopulmonary Toxicity

Two experiments were undertaken to examine the possible role of 5HT in MCTP-induced cardiopulmonary toxicity. Platelets compete with lung endothelium for uptake of 5HT (Steinberg and Das, 1980), and removal of 5HT is depressed in isolated lungs from MCTP-treated rats (Hilliker et al., 1983a). As a result of this, platelets from MCTP-treated rats may store more 5HT than platelets from control rats. Accordingly, the concentration of 5HT in platelets from MCTP-treated rats was compared to that in platelets from control rats. In addition, the effect of co-treatment with a 5HT receptor antagonist on MCTP toxicity was determined.

A. Determination of Platelet 5HT

Rats were treated with MCTP (3.5 mg/kg) or DMF on Day 0, and were killed on Day 14. Arterial blood (5 ml) was collected from ether-anesthetized rats, and PRP was prepared as described in Methods. The concentration of 5HT was determined in platelet-poor plasma and in the supernatant from sonicated, washed platelet pellets by HPLC. HPLC analysis was performed by Nancy J. Shannon.

Fourteen days after treatment with MCTP, lung weight was elevated and lavage protein concentration was higher in treated rats compared to controls (Table 16). MCTP treatment also caused RVE.

Platelet number was not different in PRP prepared from the blood of MCTP-treated rats compared to controls (Table 17). There was also no difference in the platelet concentration of protein. The 5HT concentration in PPP from

TABLE 16

MCTP Toxicity in Rats used to Determine Platelet 5HT Content

	Treatment[a]	
	DMF	MCTP
WL/BW (x1000)	3.9±0.3	9.1±0.8[b]
Lavage protein (mg/ml)	0.11±0.03	2.07±0.28[b]
RV/(LV+S)	0.272±0.007	0.399±0.026[b]

[a]Rats were treated on Day 0 with MCTP (3.5 mg/kg) or DMF and were killed 14 days later. WL = wet lung weight; BW = body weight; RV = right ventricular weight; LV+S = weight of left ventricle plus septum. N = 5-7.

[b]Significantly different from DMF control.

TABLE 17

Effect of MCTP Treatment on Platelet Number and Platelet Protein Concentration in PRP

	Treatment[a]	
	DMF	MCTP
Platelet Number ($x10^5$)	8.5±1.1	8.4±1.0
Platelet Protein (mg/ml PRP)	0.45±0.10	0.33±0.10

[a]Rats were treated as described in Table 16 and PRP was collected as described in METHODS. N = 5-7.

treated rats was not different from controls (Figure 17B). The platelet content of 5HT was also not different in treated and control rats (Figure 17A).

B. Effect of Co-treatment with Ketanserin

In this study, rats were treated with MCTP (4 mg/kg) or DMF, and were co-treated with ketanserin (KET) (2.5 mg/kg) or the vehicle (VEH) (distilled water) orally twice daily. The purpose was to determine if KET, a 5HT receptor antagonist, would protect against the cardiopulmonary effects of MCTP. The effectiveness of this dosing regimen to antagonize platelet and vascular 5HT receptors was confirmed.

1. Confirmation of drug effect

a. 5HT-induced platelet shape change. 5HT produces a change in the transmittance of light (shape change) in rat PRP (Drummond and Gordon, 1975; Laubscher and Pletscher, 1979), and this is inhibited in vitro by KET (Lampagnani and DeGaetano, 1982). To determine if the platelet receptors responsible for the 5HT-induced shape change were effectively blocked at this dose of KET (2.5 mg/kg orally, twice daily), blood was collected from rats treated with KET or the vehicle at various times after treatment. PRP was harvested from the blood, and the shape change in response to 5HT was determined.

In PRP from control rats, addition of 5HT (0.1-2 μg) dissolved in Tris-HCl buffer consistently resulted in a change in the transmittance of light (shape change) subsequent to a shift in the baseline. A typical recorder tracing from the platelet aggregometer is shown in Figure 18A. Addition of Tris-HCl buffer alone produced only a shift in the baseline (Figure 18B). When rats were treated with KET (2.5 mg/kg, orally) twice daily for 4 days, the shape change in response to 5HT (1 μg) was abolished (Figure 18C) at both 6 and 12 hours after the final KET dose. After treatment for 14 days, the change in light

Figure 17. 5HT in (A) platelets and in (B) PPP from rats treated 14 days earlier with MCTP (3.5 mg/kg) or DMF. Platelets were collected and 5HT was determined by HPLC as described in METHODS. N = 5-7.

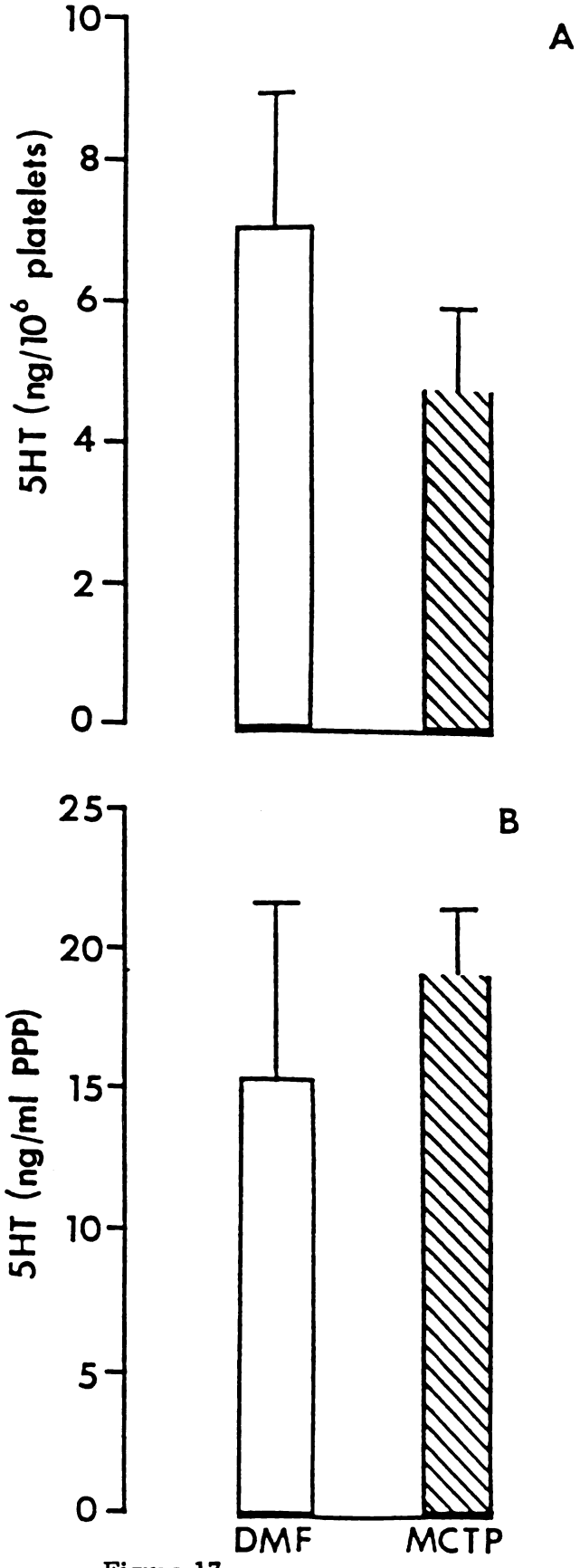
A
5HT (ng/10⁶ platelets)
10
8
6
4
2
0
B
5HT (ng/ml PPP)
25
20
15
10
5
0
DMF
MCTP

Figure 17

Figure 18. 5HT-induced shape change in PRP. Each tracing depicts the change in light transmittance in PRP over time. Panels represent typical responses to (A) 2 μg of 5HT in PRP from a control rat, (B) Tris-HCl buffer in PRP from a control rat and (C) 1 μg of 5HT in PRP from a rat treated twice daily with ketanserin (2.5 mg/kg, p.o.) for 4 days. Arrows indicate point of addition of 5HT or buffer.

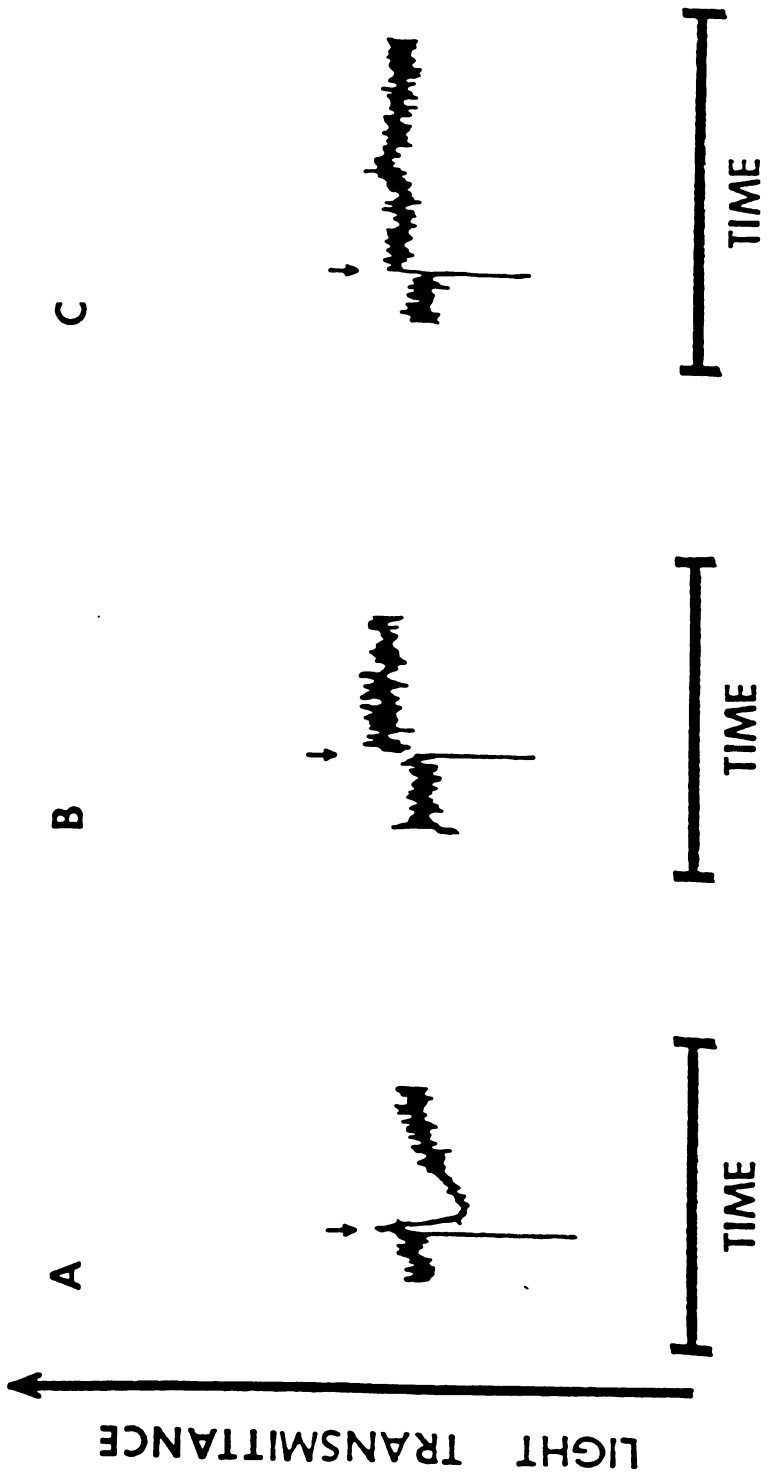

Figure 18

transmittance in PRP from blood collected 12 hours following the last administration of KET was decreased in magnitude to 24% of control (Figure 19).

b. 5HT-induced vascular response in the isolated, perfused lung. To determine if this dosing regimen of KET had also effectively inhibited 5HT receptors responsible for pulmonary vasoconstriction, the response to 5HT in isolated, perfused lungs from MCTP-treated rats was examined. Rats were co-treated with KET (2.5 mg/kg, orally twice daily) or the vehicle starting 3 days after MCTP treatment and continuing through Day 14, and lungs were isolated and perfused with blood from MCTP-treated rats co-treated with KET or the vehicle, respectively. Perfusion pressure was monitored, and the increase in pressure in response to 5HT (50 μg) was recorded. To determine the specificity of receptor antagonism, the increase in perfusion pressure in response to angiotensin II (AII, 0.5 μg) was also recorded.

Baseline perfusion pressure was the same in isolated lungs from MCTP-treated rats co-treated with either the vehicle (22±2 mmHg) or KET (22±2 mmHg). The response to AII was not significantly altered by co-treatment with KET (Figure 20). However, 10-12 hours after the final dose of KET (i.e., at the end of a dosing interval) the response to a relatively large dose of 5HT (50 μg, Hilliker and Roth, 1985a) was depressed by 56% in lungs from KET-treated rats.

2. Effect of ketanserin on MCTP-induced cardipulmonary toxicity

KET treatment alone did not affect body weight gain (Table 18). MCTP-treated rats gained less weight than control rats, and there was no significant difference between final body weight in MCTP-treated rats which received KET and those that received the vehicle.

Wet lung weight was increased in MCTP-treated rats, and this increase was not affected by treatment with KET (Figure 21A). There was no difference in the ratio of ^{125}I lung/^{125}I blood measured for the KET/DMF group

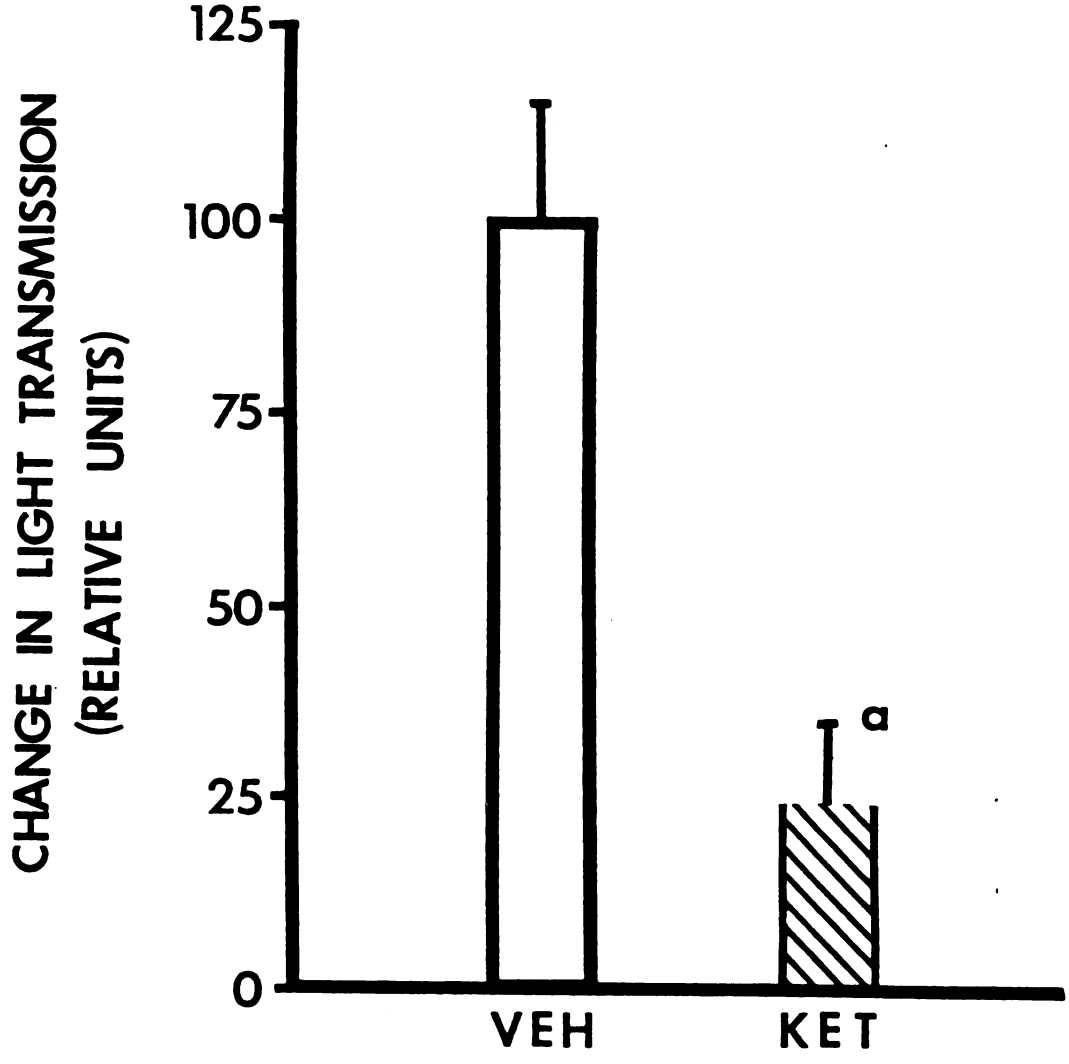

Figure 19. Effect of ketanserin (KET) treatment on 5HT-induced shape change in PRP. Rats were treated with KET (2.5 mg/kg, p.o.) or the water vehicle (VEH) twice daily for 14 days. Twelve hours after the final administration, blood was collected, and the shape change in PRP in response to addition of 1 μg of 5 HT was measured using platelet aggregometry. N = 6-7. a = significantly different from control.

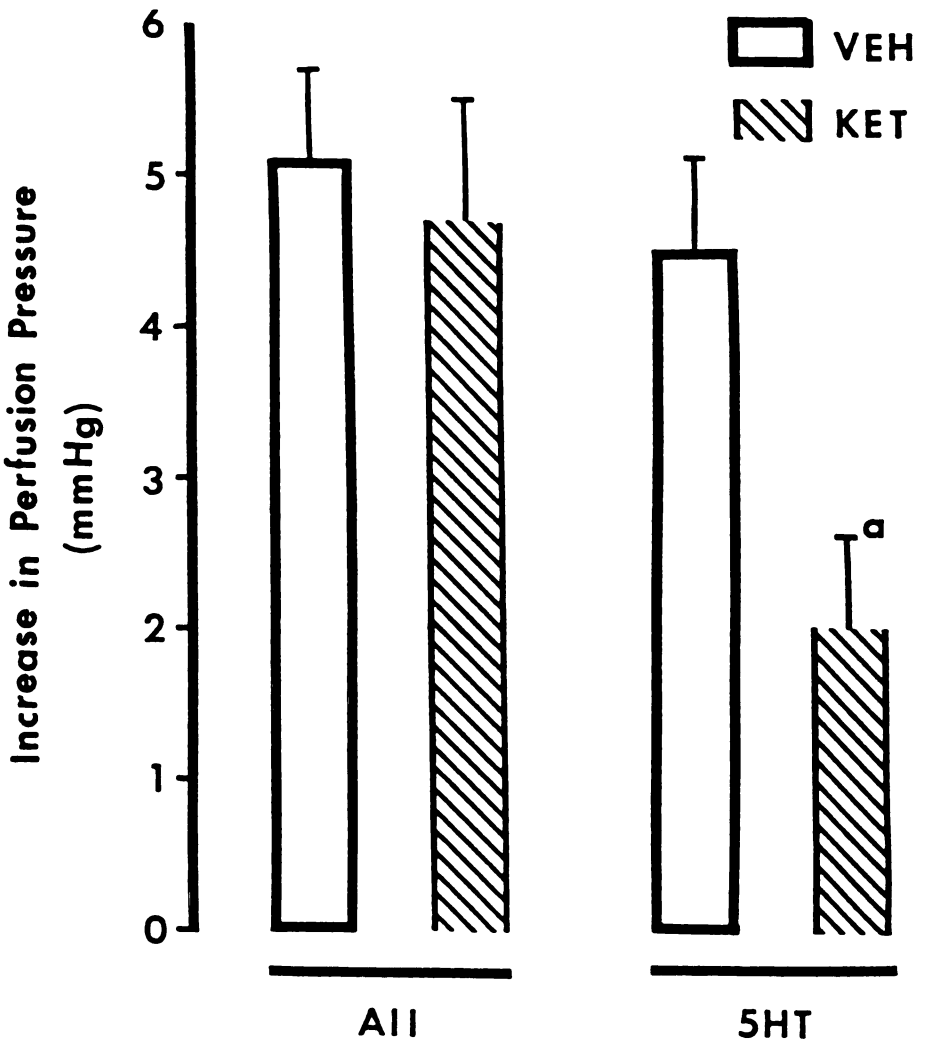

Figure 20. Effect of co-treatment with ketanserin on the response to 5HT in isolated, perfused lungs from MCTP-treated rats. On day 0, rats were treated with MCTP (4 mg/kg). Starting on day 3 and continuing through day 14, rats received VEH or KET (2.5 mg/kg) orally twice daily. Blood was collected, and lungs were isolated and perfused 10-12 hours after the final dose of KET. Inflow perfusion pressure was recorded in response to infusion of AII (0.50 μg) followed by 5HT (50 μg). N = 4-5. a = significantly different from control.

TABLE 18

Effect of Ketanserin on Body Weight of MCTP-Treated Rats

Treatment[a]	VEH/DMF	KET/DMF	VEH/MCTP	KET/MCTP
BW, Initial (g)	206±10	193±8	209± 9	204± 8
BW, Final (g)	302±12	274±4	252±20[b]	236±10
ΔBW (g)	96± 5	81±8	44±14[b]	40± 8[c]

[a]Rats were treated with MCTP (4 mg/kg) or DMF. Starting on day 3 after MCTP administration, rats received ketanserin (KET) (2.5 mg/kg) or water (VEH) twice daily by oral gavage. BW = body weight. N = 5-7.

[b]Significantly different from VEH/DMF.

[c]Significantly different from KET/DMF.

Figure 21. Effect of KET on (A) lung weight, (B) vascular leak, and (C) right ventricular enlargement in MCTP-treated rats. On day 0, rats were treated with MCTP (4 mg/kg) or DMF. Starting on day 3 and continuing through day 14, rats received KET (2.5 mg/kg) orally, twice daily. N = 5-7. a = significantly different from VEH/DMF. b = significantly different from KET/DMF. c = significantly different from VEH/MCTP.

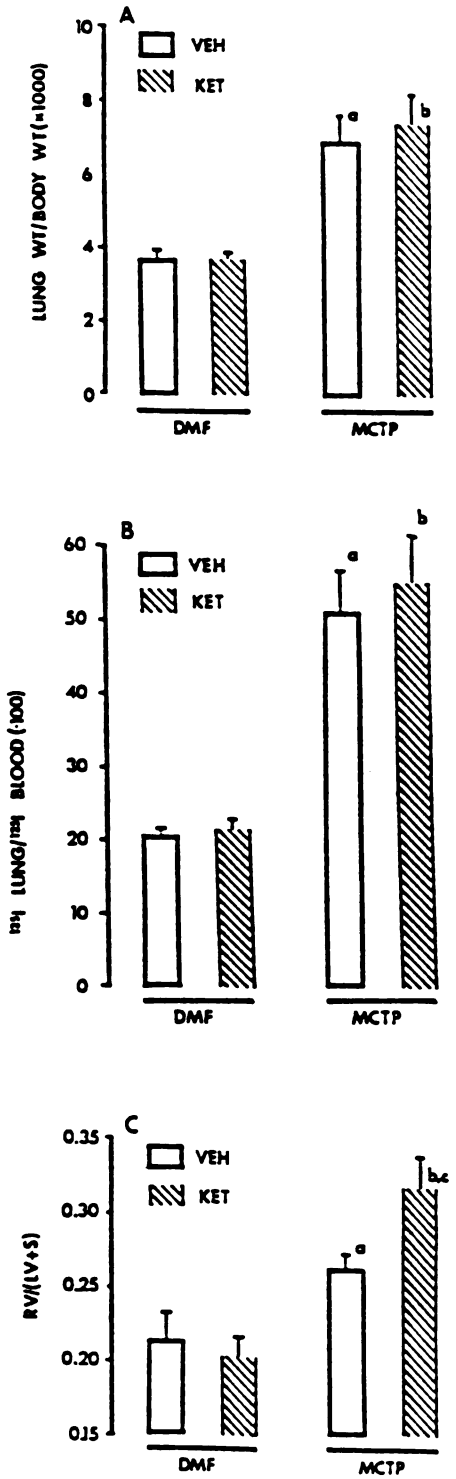
A
VEH
KET
LUNG WT/BODY WT(=1000)
10
8
6
4
2
0
a
b
DMF
MCTP
B
VEH
KET
^{125}I LUNG/^{125}I BLOOD (=100)
60
50
40
30
20
10
0
a
b
DMF
MCTP
C
VEH
KET
RV/(LV+S)
0.35
0.30
0.25
0.20
0.15
a
b,c
DMF
MCTP

Figure 21

and that of the VEH/DMF group, indicating that KET treatment alone did not affect this index of lung injury (Figure 21B). There was a significant increase in the accumulation of ^{125}I-albumin in the lungs of MCTP-treated rats, and co-treatment with KET did not affect this increase. In control rats, KET alone did not affect the RV/(LV+S) ratio (Figure 21C). MCTP treatment resulted in RVE, which was higher in KET-treated rats.

The results of these studies indicate that MCTP treatment does not alter platelet 5HT content and that antagonism of 5HT-receptors does not attenuate the cardiopulmonary response to MCTP.

VI. Examination of the Role of Thromboxane in MCTP-induced Cardiopulmonary Toxicity

Several experiments were undertaken to examine the possibility that thromboxane may contribute to MCTP-induced pulmonary hypertension. The vasoconstrictory and pro-aggregatory activities of thromboxane A_2 (TxA_2) are opposed in vivo by the vasodilatory and anti-aggregatory activities of prostacyclin (PGI_2) (Gryglewski et al., 1976). PGI_2 is primarily synthesized by endothelium, and because MCTP causes endothelial cell damage, it seemed possible that PGI_2 production could be altered in lungs of MCTP-treated rats. If PGI_2 production were decreased and/or TxA_2 production was increased in the lung, this would favor the vasoconstrictive effects of thromboxane, and could contribute to pulmonary hypertension. To examine this, the release of stable metabolites of PGI_2 and TxA_2 were determined in isolated, perfused lungs from MCTP-treated rats.

Another mechanism by which TxA_2 could contribute to MCTP-induced pulmonary hypertension is by increased synthesis and/or release of TxA_2 by platelets. To determine if MCTP treatment altered platelet synthesis or release

of Tx, TxB_2 was compared in PRP from treated and control rats. TxB_2 was measured prior to and during aggregation of platelets with arachidonic acid (AA).

Finally, the ability of drugs which interfere with Tx synthesis or activity to alter the response to MCTP was examined. The effect of co-treatment with either a cyclo-oxygenase inhibitor, a thromboxane synthetase inhibitor, or a thromboxane receptor antagonist was determined.

A. Prostanoid Release in Isolated, Perfused Lungs

To examine whether MCTP treatment in vivo altered prostanoid production or release by the lung, isolated lungs were perfused, and the concentrations of 6-keto $PGF_{1\alpha}$ (a stable metabolite of PGL_2) and TxB_2 (a stable metabolite of TxA_2) in the effluent perfusion medium were determined. Prostanoid release was determined at a time early during the development of pulmonary hypertension (day 7) and when pulmonary hypertension was well-established (day 14). In separate experiments isolated lungs were perfused with either a buffer or with blood.

1. Release in lungs perfused with buffer

Isolated lungs from rats treated 7 or 14 days earlier with MCTP (4 mg/kg) or DMF were perfused with a Krebs-bicarbonate buffer. The purpose was to determine prostanoid release from the lung when the perfusion medium did not contain cells which could contribute to prostanoid production. In addition, lungs from rats treated 14 days earlier with MCTP were challenged with arachidonic acid to examine whether MCTP treatment affected the lungs maximal capacity to synthesize 6-keto $PGF_{1\alpha}$ or TxB_2.

a. Day 7 after MCTP treatment. Lungs from rats treated 7 days earlier with MCTP or DMF were isolated and perfused with Krebs-bicarbonate buffer containing 4% bovine serum albumin (BSA). Effluent samples

were collected periodically during the 30 minutes of perfusion, and the concentrations of 6-keto $PGF_{1\alpha}$ and TxB_2 in the effluent were determined by RIA.

Rats treated 7 days earlier with MCTP gained less weight than controls (Table 19). The wet lung weight in MCTP-treated animals was elevated relative to body weight or relative to dry lung weight, indicating fluid accumulation in these lungs. There was also a greater activity of the cytoplasmic enzyme LDH in the cell-free bronchoalveolar lavage fluid from treated rats than in that of controls.

There were no differences in the release of either 6-keto $PGF_{1\alpha}$ or TxB_2 in lungs from control and MCTP-treated rats at this time (Figure 22). The concentration of TxB_2 was low throughout the 30 minutes of perfusion, hovering near the detection limit of the RIA (0.025 ng/ml) after 10 minutes in lungs from MCTP-treated rats and after 20 minutes in controls. The concentration of 6-keto $PGF_{1\alpha}$ was initially high in both groups and soon decreased, leveling off after 20 minutes of perfusion. This initial release of 6-keto $PGF_{1\alpha}$ was not seen in indomethacin-treated lungs; in fact, in these lungs, the concentration of 6-keto $PGF_{1\alpha}$ in the effluent was at or below the limit of sensitivity of the RIA throughout the perfusion. Based on these data, lungs in subsequent studies were pre-perfused for 15 minutes to allow for this initial release of 6-keto $PGF_{1\alpha}$.

Inflow perfusion pressure was higher in lungs from MCTP-treated rats at the beginning and the end of the perfusion, however the increase in pressure during perfusion was not different from controls (Table 20).

b. Arachidonic acid concentration/response relation. To determine how lungs from MCTP-treated rats would respond to an AA challenge, AA was introduced directly into the pulmonary arterial cannula. In preliminary experiments using lungs from untreated rats, different concentrations of AA were

TABLE 19

MCTP Toxicity at Day 7 in Rats used in Isolated, Buffer-Perfused Lung Studies

	Treatment[a]	
	DMF	MCTP
$BW_{initial}$ (g)	224±4	220±3
BW_{final} (g)	280±3	240±10[b]
WL/BW (x1000)	3.8±0.3	7.8±0.7[b]
WL/DL	4.7±0.3	6.9±0.4[b]
LDH activity (U/dl)	3.6±0.3	24.7±6.2[b]

[a]Rats were treated with MCTP (4 mg/kg) or DMF on day 0 and were killed on day 7. BW = body weight; WL = wet lung weight; DL = dry lung weight. N = 4-5.

[b]Significantly different from DMF.

Figure 22. Release of (A) 6-keto $PGF_{1\alpha}$ and (B) TxB_2 into effluent perfusion medium from isolated lungs of rats treated 7 days earlier with DMF control (solid lines) or MCTP (broken lines). Lungs of rats treated 7 days earlier with MCTP or DMF vehicle were isolated and perfused in a single-pass system with Krebs-bicarbonate buffer containing 4% BSA. Effluent was collected at various times during the perfusion, and prostanoid concentrations were determined by radioimmunoassay (RIA). N = 4-5.

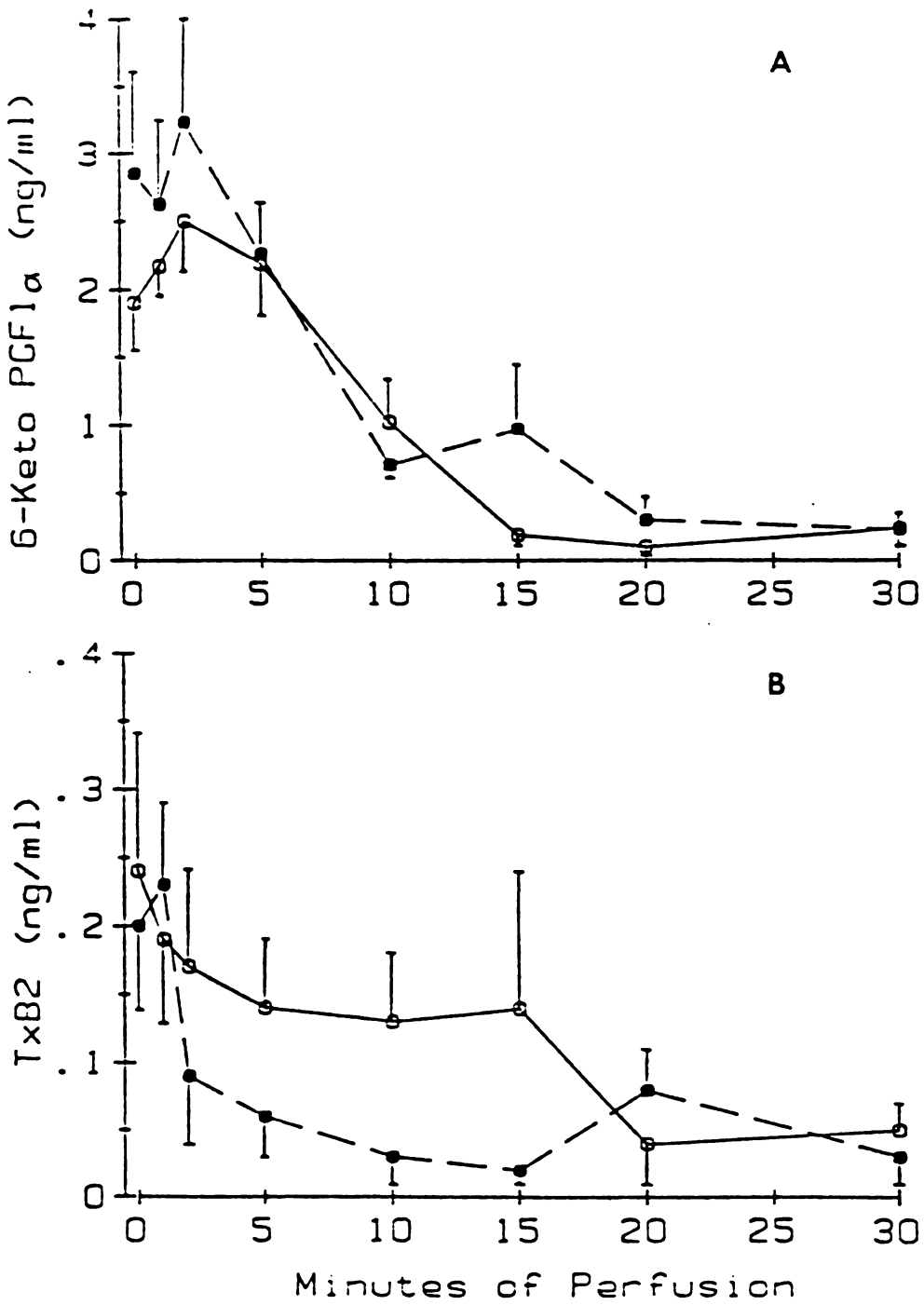
A
6-Keto PGF1α (ng/ml)
0
1
2
3
0
5
10
15
20
25
30
B
TxB2 (ng/ml)
.1
.2
.3
.4
Minutes of Perfusion

Figure 22

TABLE 20

Inflow Perfusion Pressure in Isolated Lungs from Rats Treated 7 Days Earlier with MCTP

	Treatment[a]	
	DMF	MCTP
$PP_{initial}$ (mmHg)	4.7±0.2	6.2±0.3[b]
PP_{final} (mmHg)	5.6±0.3	7.5±0.4[b]
ΔPP (mmHg)	0.9±0.2	1.3±0.2

[a]Rats were treated and lungs were perfused as described in the legend to Figure 22. PP = inflow perfusion pressure. N = 4-5.

[b]Significantly different from DMF controls.

infused into the pulmonary artery at 0.1 ml/min to determine a concentration of AA which would result in increased release of 6-keto $PGF_{1\alpha}$. The lungs were preperfused with a Krebs-bicarbonate buffer containing 4% BSA, then were perfused with a BSA-free Krebs-bicarbonate buffer. Removal of the BSA was necessary because AA binds to albumin, and in the presence of BSA only very high concentrations of AA caused increased release of 6-keto $PGF_{1\alpha}$, and this increase was not reproducible among perfusions.

AA at 40 μM did not stimulate release of 6-keto $PGF_{1\alpha}$ (Figure 23). At 120 μM AA, the lung became very edematous, and the perfusion was terminated. An intermediate concentration of 80 μM AA stimulated release of 6-keto $PGF_{1\alpha}$, and yet the lungs did not become edematous. This concentration was used in subsequent studies.

c. Day 14 after MCTP treatment. Fourteen days after treatment with MCTP or DMF, rats were killed, the lungs were isolated and perfused, and lung weight and RVE were determined. The lungs were perfused as described above for the arachidonic acid concentration/response relation. Samples of the effluent were collected every other minute starting 12 minutes after switching to the BSA-free perfusion medium. In a separate experiment, lungs were purposely made edematous, and the effect of edema formation on the release of 6-keto $PGF_{1\alpha}$ and TxB_2 from isolated lungs was examined.

Body weight gain was suppressed and wet lung weight was elevated relative to body weight and relative to dry lung weight 14 days after treatment with MCTP (Table 21). In addition, RVE was evident.

The release of 6-keto $PGF_{1\alpha}$ was not different in lungs from control and MCTP-treated animals prior to or during infusion of AA (Figure 24A). The release of 6-keto $PGF_{1\alpha}$ increased in response to AA in lungs from both treated and control animals.

Figure 23. Release of 6-keto $PGF_{1\alpha}$ from isolated, perfused lungs in response to several concentrations of arachidonic acid. Lungs from untreated rats were perfused with a BSA-free Krebs bicarbonate buffer as described in METHODS. Arachidonic acid infusion (0.1 ml/min) into the pulmonary arterial cannula began at 13 minutes, and each lung received only one concentration of arachidonic acid: ■-·-■, 120 μM, n=1; o—o, 80 μM, n=5; ◆— —◆, 40 μM, n=7.

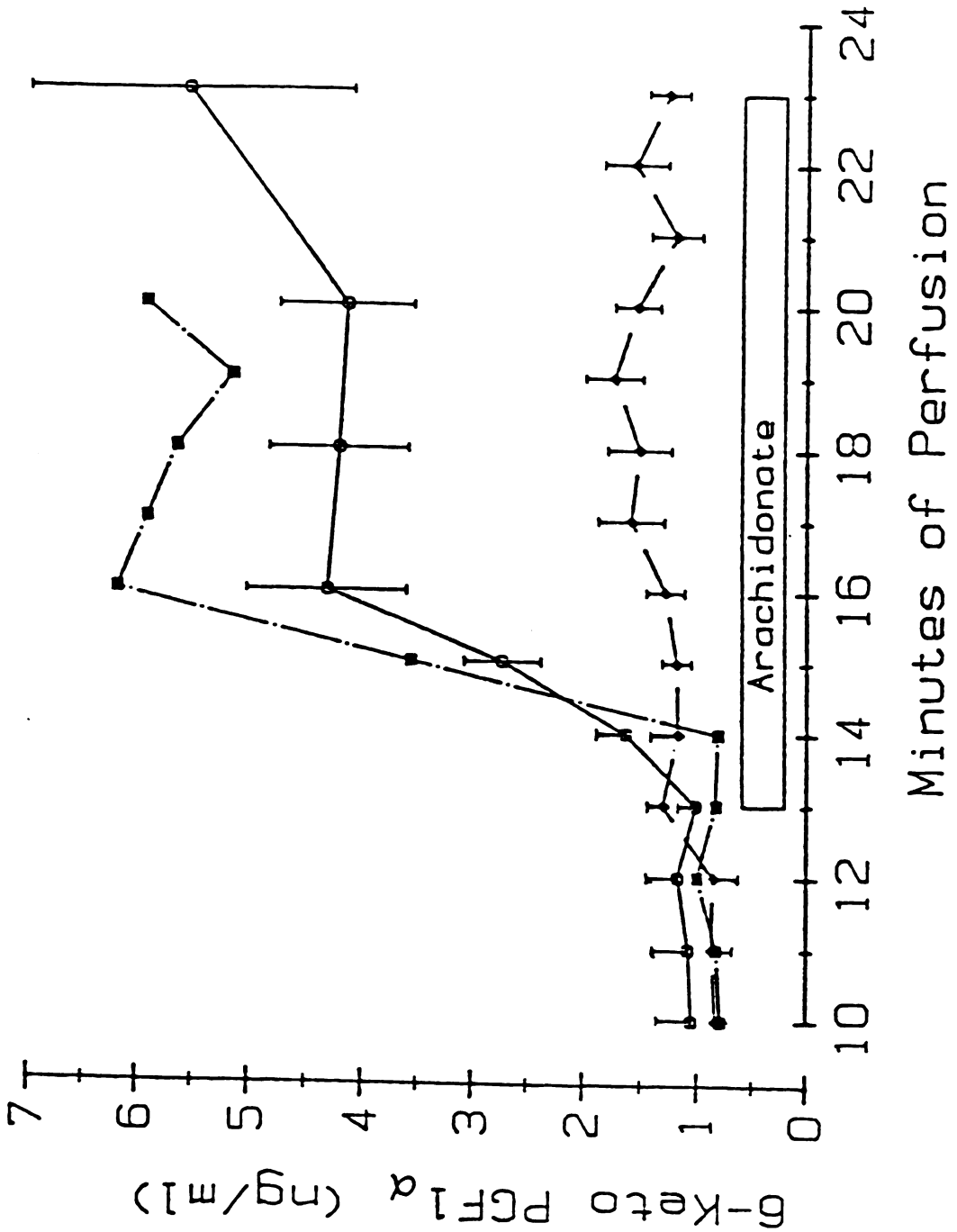
6-Keto PGF1α (ng/ml)
0
1
2
3
4
5
6
7
10
12
14
16
18
20
22
24
Minutes of Perfusion
Arachidonate

Figure 23

TABLE 21

MCTP Toxicity at Day 14 in Rats used in Isolated, Buffer-Perfused Lung Studies

	Treatment[a]	
	DMF	MCTP
$BW_{initial}$ (g)	238±11	250±15
BW_{final} (g)	338±7	253±14[b]
WL/BW (x1000)	4.1±0.2	13.9±2.0[b]
WL/DL	6.0±0.2	9.6±0.9[b]
RV/(LV+S)	0.261±0.005	0.363±0.016[b]

[a]Rats received DMF or MCTP (4 mg/kg, i.v.) on day 0 and were killed on day 14. BW = body weight; WL = wet lung weight; DL = dry lung weight. RV/(LV+S) = right ventricular weight/weight of left ventricle plus septum. N=8.

[b]Significantly different from DMF controls.

Figure 24. Release of (A) 6-keto $PGF_{1\alpha}$ and (B) TxB_2 into the effluent of lungs isolated from control (solid lines) and MCTP-treated rats (broken lines) 14 days after treatment. The lungs were perfused with Krebs bicarbonate buffer, and at 13 minutes arachidonic acid (80 μM, 0.1 ml/min) infusion into the pulmonary arterial cannula was begun. Samples were collected and prostanoid concentrations were determined by RIA. N=8. a = values significantly different from the value at 12 minutes for the same group. b = significant differences between groups at the times indicated.

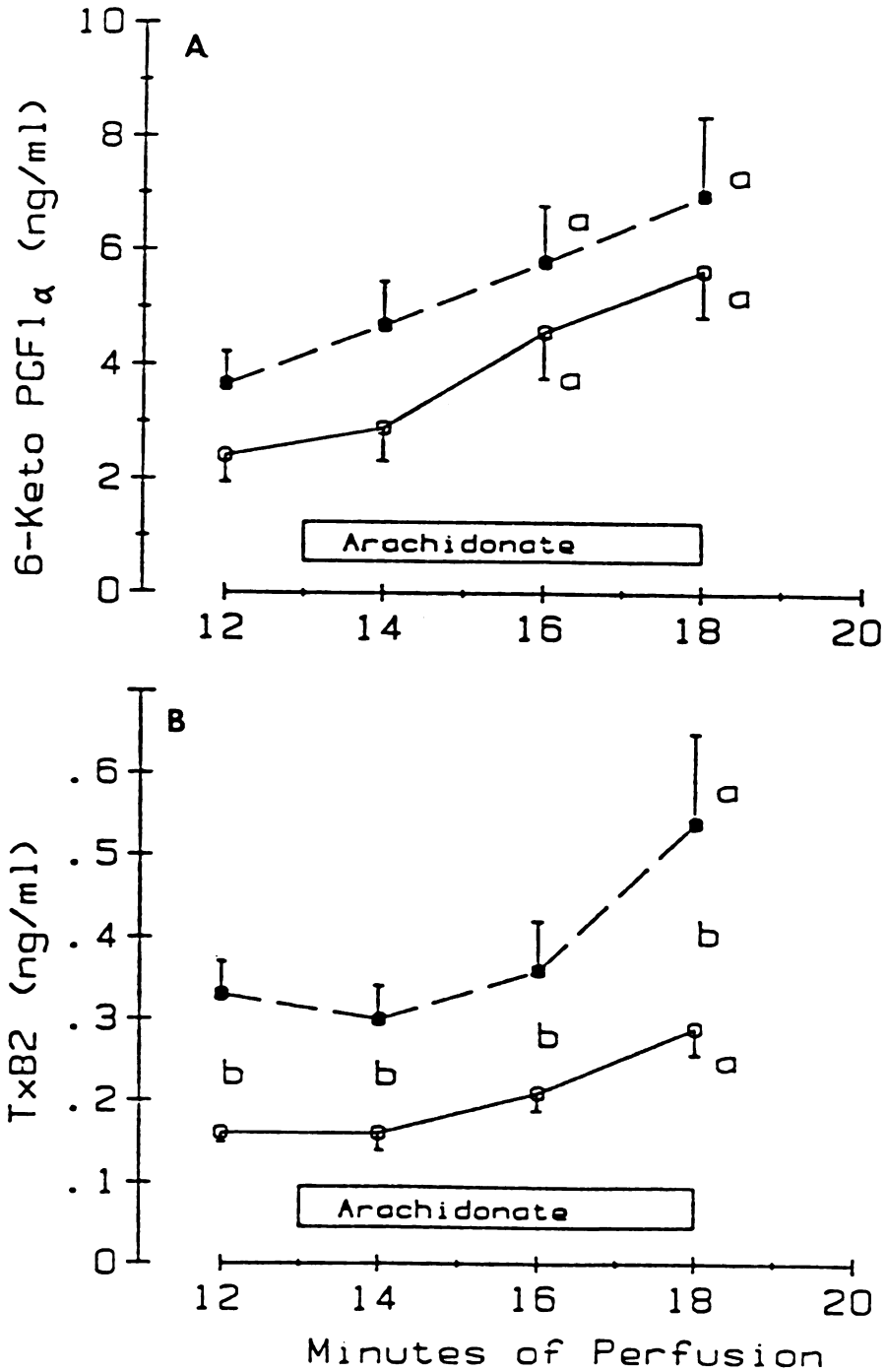
A
6-Keto PGF1α (ng/ml)
10
8
6
4
2
0
a
a
a
a
Arachidonate
12
14
16
18
20
B
TxB2 (ng/ml)
.6
.5
.4
.3
.2
.1
0
a
b
b
b
b
a
Arachidonate
12
14
16
18
20
Minutes of Perfusion

Figure 24

TxB_2 release increased during AA infusion in lungs from both groups of animals (Figure 24B). The concentration of TxB_2 was higher in the effluent of lungs from MCTP-treated rats than controls both before and during AA infusion.

Inflow perfusion pressure was significantly higher in lungs from MCTP-treated rats initially and at the end of the perfusion (Table 22). However, the increase in pressure during the perfusion was not different in the two groups.

Because isolated lungs from MCTP-treated rats released more TxB_2 than lungs from control rats at day 14, and because these lungs were also more edematous than control lungs (as evidenced by an increase in wet lung weight, Table 21), the effect of edema formation on the release of 6-keto $PGF_{1\alpha}$ and TxB_2 was examined. The mean lung weight/body weight (x1000) following perfusion at increased outflow pressure was 12.8 ± 1.6 (n=3), similar to that seen in isolated, perfused lungs from rats treated 14 days earlier with MCTP (Table 21). The release of 6-keto $PGF_{1\alpha}$ from lungs made edematous by increasing outflow pressure was similar to that seen in lungs from rats treated with MCTP or DMF 14 days earlier, and it did not change during the perfusion (Figure 25A, compare to Figure 24A). The release of TxB_2 from edematous lungs was relatively high initially, close to the concentration seen in lungs from MCTP-treated rats, but leveled off by 16 minutes of perfusion to a concentration similar to that seen in control lungs (Figure 25B, compare to Figure 24B). The concentration of TxB_2 in the effluent at 10 minutes was not significantly different from the concentration at 20 minutes. This suggests that the propensity for lungs from MCTP-treated rats to take on fluid may contribute to increased TxB_2 release.

TABLE 22

Inflow Perfusion Pressure in Lungs Isolated from Rats Treated 14 Days Earlier with MCTP

	Treatment[a]	
	DMF	MCTP
$PP_{initial}$ (mmHg)	5.8±0.5	8.0±0.6[b]
PP_{final} (mmHg)	6.5±0.4	11.0±1.4[b]
ΔPP (mmHg)	0.8±0.4	3.0±1.1

[a]Rats were treated and lungs were perfused as described in the legend to Figure 24. PP = inflow perfusion pressure. N=8.

[b]Significantly different from DMF controls.

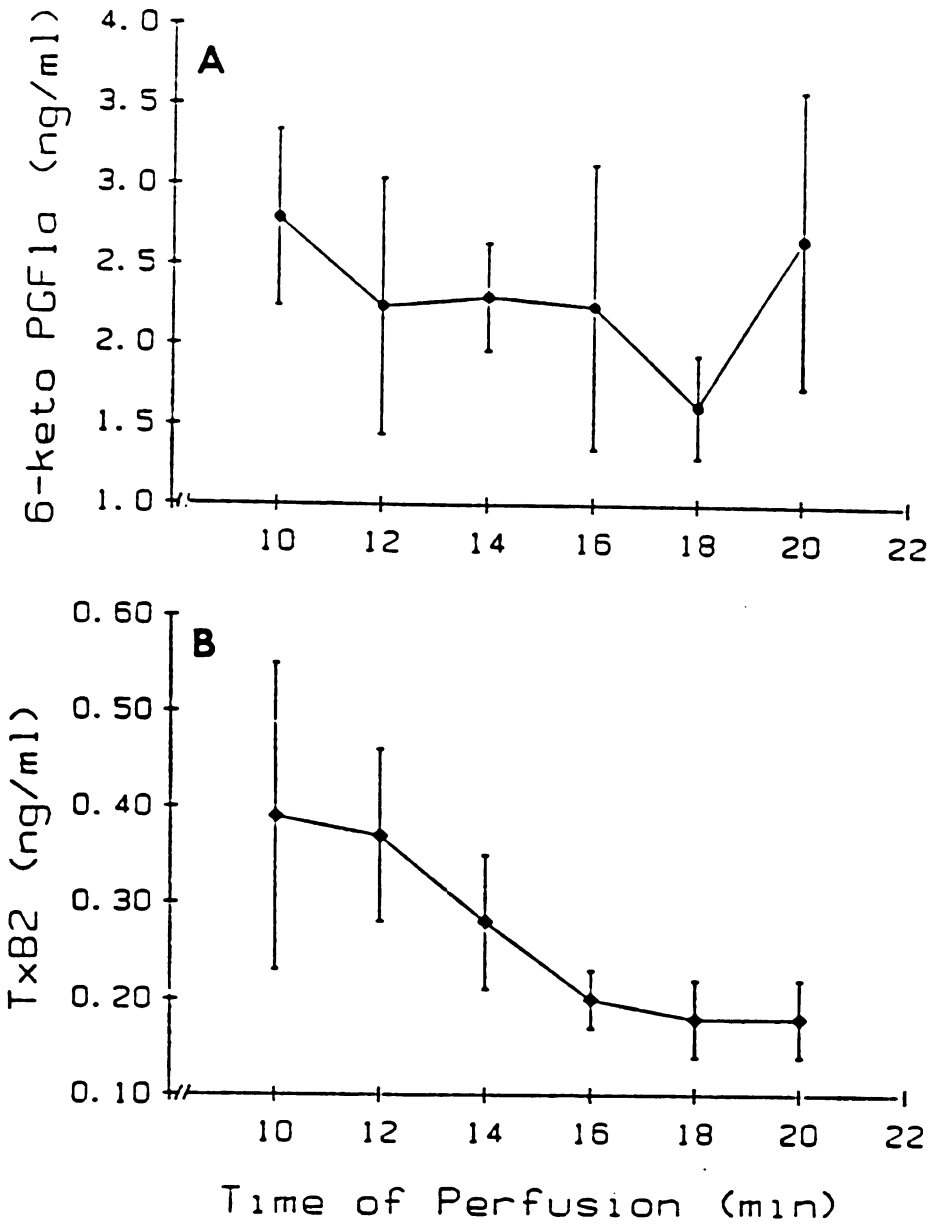

Figure 25. Release of (A) 6-keto $PGF_{1\alpha}$ and (B) TxB_2 in isolated lungs made edematous. Lungs from untreated rats were perfused with Krebs bicarbonate buffer at a flow of 40 ml/min. The left atrial cannula was elevated so that outflow pressure was 8 cm H_2O. When the lungs appeared to have taken on fluid, outflow pressure was returned to 0 cm H_2O and flow was decreased to 8 ml/min. After 10 min, effluent samples were collected every other minute. Prostanoid concentrations were determined by RIA. N=3.

2. Release in lungs perfused with blood

The results of the above studies in which isolated lungs were perfused with buffer indicated that release of TxB_2 but not 6-keto $PGF_{1\alpha}$ was greater in lungs from MCTP-treated rats than controls later (day 14) in the development of pulmonary hypertension. Therefore, it was of interest to examine this relationship in isolated lungs perfused with a platelet containing medium to investigate whether the presence of platelets would enhance the difference in TxB_2 release in lungs from treated and control rats. Although whole blood contains several cell types which can contribute to the plasma Tx concentration, whole blood was the preferred medium to maintain the platelets in the most natural environment.

Blood for the perfusions was obtained from untreated rats. Lungs from rats treated 7 or 14 days earlier with MCTP (4 mg/kg) or DMF were isolated, preperfused with a buffer, then perfused for 4 minutes with blood. Samples of the blood effluent were collected every minute and immediately spun in a centrifuge, and the concentrations of 6-keto $PGF_{1\alpha}$ and TxB_2 were determined in the plasma effluent by RIA. The concentrations of these prostanoids were also measured in the blood prior to perfusion. Lung weight and RVE were recorded as well.

a. Day 7 after treatment with MCTP. Seven days after treatment with MCTP, lung weight was significantly greater in treated animals (Table 23). Body weight gain and RV/(LV+S) were not affected by MCTP treatment at this time.

Prior to perfusion, the concentrations of TxB_2 in the blood was not significantly different in the two groups (Figure 26B). The same was true of the concentration of 6-keto $PGF_{1\alpha}$ in the blood (Figure 26A). The concentration of 6-keto $PGF_{1\alpha}$ in the plasma effluent was not significantly different in

TABLE 23

MCTP Toxicity at Day 7 in Rats used in Isolated, Blood-Perfused Lung Studies

	Treatment[a]	
	DMF	MCTP
$BW_{initial}$ (g)	241±6	241±7
BW_{final} (g)	291±7	276±6
WL/BW (x1000)	4.4±0.1	5.9±0.3[b]
RV/(LV+S)	0.23±0.01	0.24±0.01

[a]Rats received MCTP (4 mg/kg) or DMF i.v. on day 0. On day 7, rats were killed and their lungs were isolated and perfused. BW = body weight; WL = wet lung weight. N = 5-6.

[b]Significantly different from DMF control.

Figure 26. (A) 6-Keto $PGF_{1\alpha}$ and (B) TxB_2 in the plasma effluent of isolated lungs from DMF control (solid lines) and MCTP-treated (broken lines) rats 7 days after treatment. Rats were given MCTP (4 mg/kg) or DMF on day 0 and were killed on day 7. Lungs were isolated, preperfused with buffer, then perfused for 4 minutes with blood. Prostanoid concentrations in the plasma were determined by RIA. P = pre-perfusion. N = 5-6. a = significantly different from the respective pre-perfusion value.

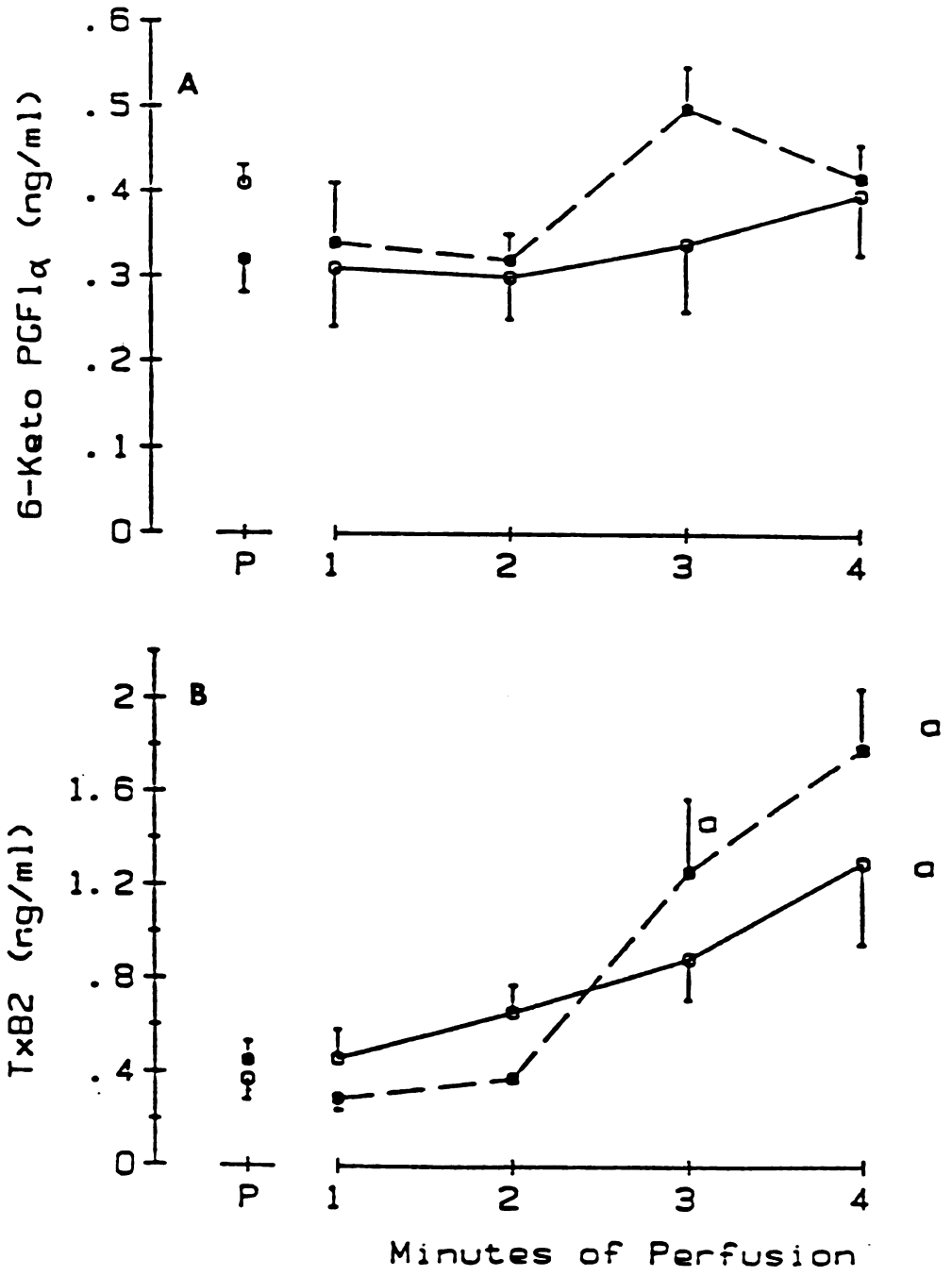
A
6-Keto PGF1α (ng/ml)
.6
.5
.4
.3
.2
.1
0
P
1
2
3
4
B
TxB2 (ng/ml)
2
1.6
1.2
.8
.4
0
a
a
a
P
1
2
3
4
Minutes of Perfusion

Figure 26

lungs from treated and control rats at any time during the perfusion, and it did not change during the period of perfusion (Figure 26A). There was no significant difference in the concentration of TxB_2 in the plasma effluent of lungs from control or MCTP-treated rats at any time during the perfusion (Figure 26B). The concentration of TxB_2 in the effluent increased as the time of perfusion increased in lungs from both control and treated rats.

The numbers of platelets in the blood perfusate and effluent were not different for lungs of MCTP-treated and control rats (Table 24). The difference in platelet number between inflow and effluent perfusate was not different from zero for either of the two groups, indicating that large numbers of perfused platelets were not adhering to the pulmonary vasculature. Perfusion pressure was not significantly higher in lungs from MCTP-treated rats at this time. Hematocrit and pH of the blood effluent at the end of the perfusion was also not different in the two groups (data not shown).

To determine any contribution to changes in prostanoid concentration by perfusing blood through the perfusion apparatus alone, similar "perfusions" were performed in the absence of a lung. The concentrations of TxB_2 and 6-keto $PGF_{1\alpha}$ in the plasma effluent of these sham perfusions were not significantly different from those in the lungs of control rats. Platelet number, hematocrit, and pH were also not different (data not shown).

b. Day 14 after treatment with MCTP. Body weight gain was suppressed 14 days after treatment with MCTP (Table 25). MCTP treatment also caused an elevation in lung weight and RVE at this time.

The plasma concentrations of TxB_2 (Figure 27B) in the blood before perfusion did not differ between lungs from control rats and those from MCTP-treated rats. The same was true for 6-keto $PGF_{1\alpha}$ (Figure 27A). The concentration of 6-keto $PGF_{1\alpha}$ was not significantly different in lungs from

TABLE 24

Perfusate Platelet Number and Inflow Perfusion Pressure in Isolated Lungs from Rats Treated 7 Days Earlier with MCTP

	Treatment[a]	
	DMF	MCTP
Pt_{inflow} (#x10^6/µl)	1.1±0.1	1.2±0.1
$Pt_{effluent}$ (#x10^6/µl)	1.2±0.1	1.0±0.1
ΔPt (#x10^6/µl)	0.07±0.03	-0.11±0.07
$PP_{initial}$ (mmHg)	12.9±0.5	14.6±0.7
PP_{final} (mmHg)	12.8±0.5	14.3±0.5
ΔPP (mmHg)	-0.08±0.3	-0.70±0.2

[a]Rats were treated and lungs were isolated and perfused as described in the legend to Figure 26. Pt = platelet; ΔPt = difference in platelet number in inflow and effluent blood; PP = perfusion pressure. N = 5-6.

TABLE 25

MCTP Toxicity at Day 14 in Rats used in Isolated, Blood-Perfused Lung Studies

	Treatment[a]	
	DMF	MCTP
$BW_{initial}$ (g)	274±13	267±12
BW_{final} (g)	349± 6	265±20[b]
WL/BW (x1000)	5.1±0.4	13.3±2.6[b]
RV/(LV+S)	0.23±0.01	0.31±0.02[b]

[a]Rats received MCTP (4 mg/kg) or DMF i.v. on day 0. On day 14, rats were killed and their lungs were isolated and perfused. BW = body weight; WL = wet lung weight. N = 7.

[b]Significantly different from DMF control.

Figure 27. (A) 6-Keto $PGF_{1\alpha}$ and (B) TxB_2 in the plasma effluent of isolated lungs from DMF control (solid lines) and MCTP-treated (broken lines) rats 14 days after treatment. Rats were treated i.v. with MCTP (4 mg/kg) or DMF on day 0 and were killed 14 days later. Lungs were isolated and perfused as described in the legend to Figure 26. N = 6-7. P = pre-perfusion. a = significantly different from the respective pre-perfusion value. b = significant difference between MCTP and DMF at the indicated time.

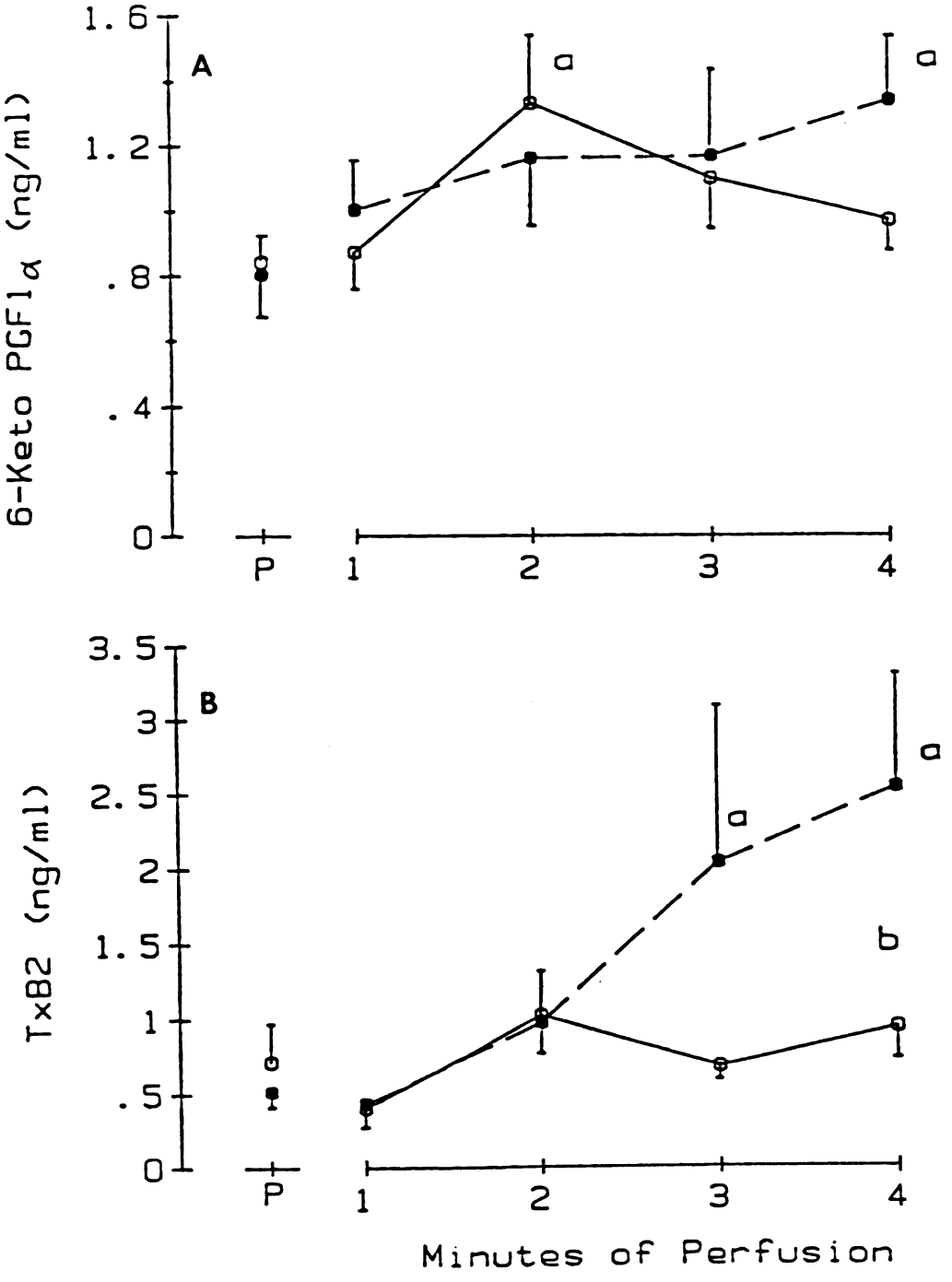
6-Keto PGF1α (ng/ml)
1.6
1.2
.8
.4
0
A
a
a
P
1
2
3
4
TxB2 (ng/ml)
3.5
3
2.5
2
1.5
1
.5
0
B
a
a
b
P
1
2
3
4
Minutes of Perfusion

Figure 27

control and MCTP-treated rats at any time during the perfusion. The concentration of TxB_2 did not change significantly during perfusion of lungs from control rats (Figure 27B). However, TxB_2 increased after 2 minutes of perfusion in lungs from MCTP-treated rats. After 4 minutes of perfusion, the concentration of TxB_2 was significantly greater in the effluent of lungs from treated rats than in that of lungs from control rats.

In neither the inflow nor the effluent perfusion medium did platelet numbers differ between treatment groups (Table 26). Also, the difference between the platelet numbers in the inflow and effluent perfusion medium was not different from zero for lungs from either treated or control rats. Inflow perfusion pressure at the end of the perfusion was higher in lungs from MCTP-treated rats than in lungs from control rats. Hematocrit and pH of the blood effluent after perfusion through the lung was not different between the two groups (data not shown).

Isolated lungs remove circulating TxB_2 (Robinson et al., 1982). The ability of lungs from MCTP-treated rats to extract TxB_2 from the blood was evaluated by comparing the difference between the pre-perfusion plasma concentration of TxB_2 and the plasma concentration in the effluent at 1 minute. This is depicted graphically for individual lungs in Figure 28. The extraction value ((preperfusion - 1 min)/pre-perfusion) was not significantly different for lungs from control (0.37 ± 0.07)and MCTP-treated (-0.09 ± 0.26) rats.

B. Generation of TxB_2 in Platelet-Rich Plasma

One mechanism by which the platelet may contribute to MCTP-induced pulmonary hypertension is through increased release of vasoactive mediators such as TxA_2. Accordingly, the hypothesis that MCTP treatment in vivo alters platelet release of Tx was investigated.

TABLE 26

Perfusate Platelet Number and Inflow Perfusion Pressure in Isolated Lungs from Rats Treated 14 Days Earlier with MCTP

	Treatment[a]	
	DMF	MCTP
Pt_{inflow} (#x10^6/µl)	1.0±0.04	1.0±0.06
$Pt_{effluent}$ (#x10^6/µl)	0.9±0.07	1.0±0.06
ΔPt (#x10^6/µl)	-0.1±0.05	-0.03±0.06
$PP_{initial}$ (mmHg)	15.3±0.9	23.7±4.3
PP_{final} (mmHg)	11.8±1.6	19.9±3.3[b]
ΔPP (mmHg)	-3.6±1.1	-3.3±1.3

[a]Rats were treated and lungs were isolated and perfused as described in METHODS. Pt = platelet; ΔPt = difference in platelet number in inflow and effluent blood. PP = perfusion pressure. N = 6-7.

[b]Significantly different from DMF by rank sums test ($p < 0.05$).

Figure 28. Extraction of TxB_2 by isolated lungs from rats treated 14 days earlier with MCTP or DMF. Lungs were perfused as described in the legend to Figure 26. Pre = concentration of TxB_2 in the plasma of the blood before perfusion; Post = concentration of TxB_2 in the plasma of the blood after a single pass through the lung (at 1 minute). Each line represents an individual lung perfusion. N=7.

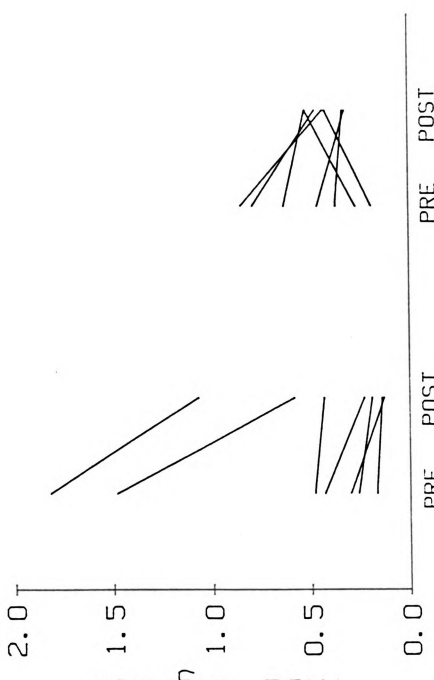

Figure 28

Rats were treated with MCTP (4 mg/kg) or DMF and were killed 1, 4, 7 or 14 days later. Blood was collected and PRP was prepared. Arachidonic acid (AA) was the stimulus for aggregation, and TxB_2 was measured in the PRP supernatant prior to and during the aggregation response (see Figure 3). The effect of MCTP in vitro on platelet aggregation and TxB_2 release was also examined. Lung weight, lavage fluid protein concentration, and RV/(LV+S) were determined at days 4, 7, and 14.

Body weight gain was suppressed in MCTP-treated rats 7 or 14 days after treatment (Table 27). Lung weight was elevated in MCTP-treated rats by day 4 and remained elevated through day 14 (Table 27). This was also true for the concentration of protein in the lavage fluid. RVE was not evident until day 14.

The aggregation response of PRP to AA was largely unaffected by MCTP treatment in vivo (Table 28). The extent of aggregation (maximal aggregation) was not different in PRP from treated and control rats at any time following treatment with MCTP, although there was a tendency toward less aggregation in PRP from treated rats at days 4 and 7. The rate of aggregation (slope) was significantly lower in PRP from treated rats at day 4, but was not different from control at any other time. The delay to aggregation was also not different in PRP from MCTP-treated and control rats.

The concentration of TxB_2 was higher in unstimulated PRP from MCTP-treated rats than control rats at day 1, but it was not different from control at any other time (Figure 29A). Comparison of the units for TxB_2 concentration in Figures 29A and 29B demonstrates that TxB_2 was released during aggregation, and most of this release had occurred by the time the platelets had reached half-maximal aggregation (compare Figures 29B and 29C). There was a small but significant decrease in the TxB_2 released at half-maximal aggregation in PRP from rats treated 7 days earlier with MCTP, but release was not different

TABLE 27

Effect of MCTP on Body Weight, Lung Weight, Lavage Fluid Protein Concentration and Right Ventricular Enlargement

Days Following Treatment	Treatment[a]	Final BW	WL/BW (x1000)	Lavage Protein (mg/ml)	RV/(LV+S)
1	DMF	250± 3	ND	ND	ND
	MCTP	248± 4	ND	ND	ND
4	DMF	297±15	3.5±0.3	0.15±0.03	0.286±0.028
	MCTP	305±15	5.4±0.6[b]	0.84±0.02[b]	0.276±0.010
7	DMF	296± 4	4.9±0.3	0.42±0.12	0.221±0.012
	MCTP	240± 8[b]	8.1±1.0[b]	2.82±0.49[b]	0.244±0.014
14	DMF	374±14	4.1±0.7	0.24±0.10	0.279±0.012
	MCTP	278±28[b]	10.1±1.5[b]	2.13±0.47[b]	0.377±0.016[b]

[a]On Day 0 rats received either MCTP (4 mg/kg) or DMF via the tail vein. BW = body weight, WL = wet lung weight. N = 3-8.

[b]Significantly different from DMF control.

ND = Not determined.

TABLE 28

Effect of MCTP Treatment In Vivo on Arachidonic Acid-induced Aggregation in Platelet-rich Plasma

Days Following Treatment	Treatment[a]	Maximal Aggregation (%)	Slope (% max/min)	Delay (minutes)
1	DMF	72± 4	29± 3	0.77±0.09
	MCTP	55±18	35±11	0.76±0.22
4	DMF	60±12	34± 7	0.69±0.02
	MCTP	40± 8	18± 1[b]	0.91±0.09
7	DMF	74± 3	29± 8	0.64±0.08
	MCTP	46±13	23± 4	0.60±0.04
14	DMF	53±11	26± 9	0.72±0.07
	MCTP	53±13	31± 9	0.67±0.08

[a]On Day 0 rats received either MCTP (4 mg/kg) or DMF via the tail vein. Blood was collected, and PRP was prepared. Arachidonic acid (1.5 mM) was used to induce aggregation. For an explanation of the parameters measured, see METHODS. N = 3-8.

[b]Significantly different from DMF control.

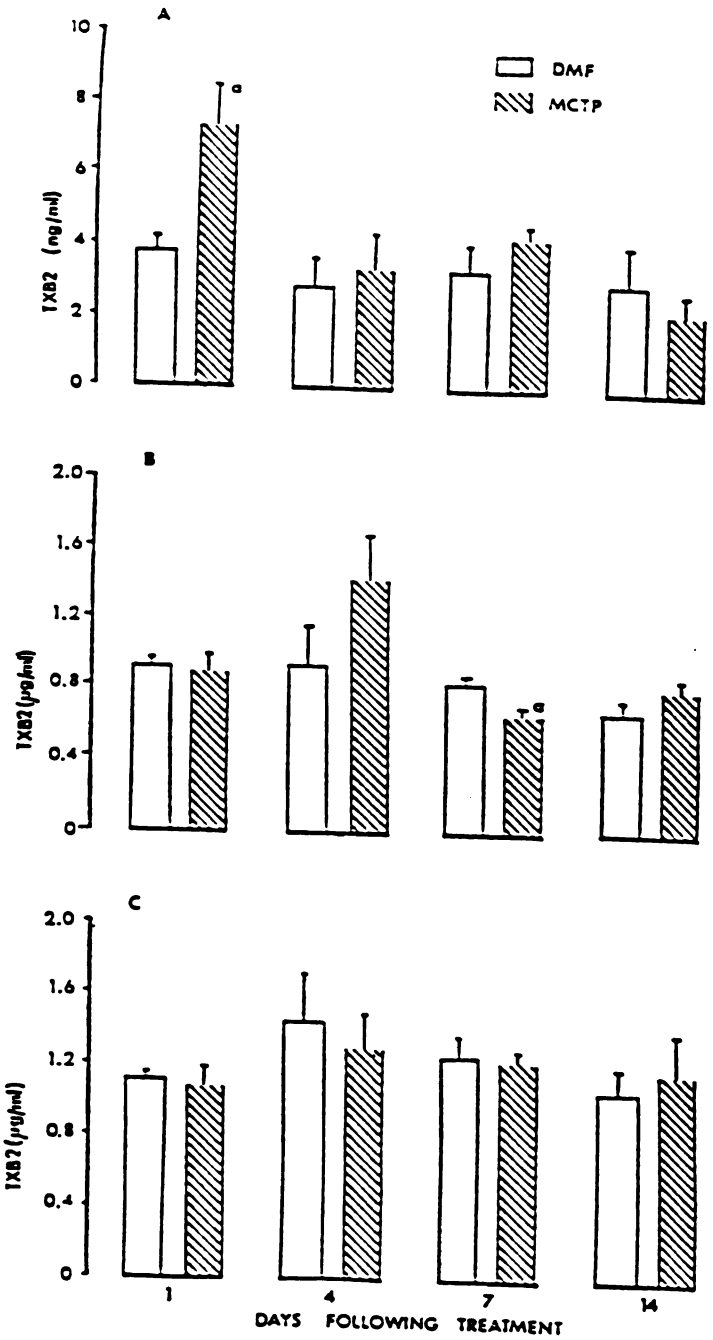

Figure 29. TxB_2 generated in (A) unstimulated PRP and in PRP at (B) half-maximal or (C) maximal aggregation with arachidonic acid (1.5 mM). Rats were treated on day 0 with MCTP (4 mg/kg) or DMF. PRP was prepared as described in METHODS and TxB_2 was measured by RIA. N = 3-8. a = significantly different from DMF control on the same day.

at any other time following treatment with MCTP (Figure 29B). The concentration of TxB_2 in PRP at maximal aggregation was not affected by treatment with MCTP (Figure 29C).

To determine if MCTP had any direct effect on platelets, MCTP was added in vitro to PRP from untreated rats, the platelets were induced to aggregate with AA, and TxB_2 generated at maximal aggregation was measured. DMF (1 µl) did not affect AA-induced aggregation or release of TxB_2 in PRP (Table 29). MCTP, in amounts up to 125 µg in 0.5 ml PRP, also had no effect on the aggregation response to AA or on the release of TxB_2. When 500 µg of MCTP was added to the PRP, the aggregation response was abolished and TxB_2 release was depressed.

The results of this study suggested that platelets from MCTP-treated rats do not respond to stimuli with enhanced release of TxB_2, and that, except at high concentrations, MCTP does not directly alter TxB_2 release or the platelet aggregation response.

C. Effect on MCTP-induced Pneumotoxicity of Drugs Which Interfere with the Synthesis or Action of TxA_2

If TxA_2 contributes to MCTP-induced pulmonary hypertension, then co-treatment with drugs which interfere with the synthesis or action of TxA_2 should attenuate the toxic response to MCTP. Two different drugs which interfere with the synthesis of TxA_2 were used. Ibuprofen inhibits the enzyme cyclooxygenase (Longenecker et al., 1985) which catalyzes the conversion of arachidonic acid to PGH_2, the cyclic endoperoxide precursor to TxA_2, PGI_2 and PG's of the A-F series. Dazmegrel inhibits thromboxane synthetase (Fischer et al., 1983), the enzyme responsible for conversion of PGH_2 to TxA_2. In addition, a Tx receptor antagonist, L-640,035, was used (Carrier et al., 1984).

TABLE 29

Effect of MCTP In Vitro on Arachidonic Acid-induced Platelet Aggregation and Release of TxB_2

Addition to PRP[a]	Maximal Aggregation (%)	Slope (% max/min)	Delay (minutes)	TxB_2 (µg/ml)
None	68±10	41±9	0.60±0.10	1.8±0.2
DMF	55±14	32±7	0.68±0.19	1.8±0.5
31 µg MCTP	29±15	17±5	0.44±0.06	1.5±0.4
62 µg MCTP	33±16	22±9	0.42±0.08	1.8±0.4
125 µg MCTP	28±12	16±5	0.38±0.09	1.3±0.2
500 µg MCTP	0±0[b]	NA	NA	0.5±0.1[b]

[a]DMF (1 µl) or MCTP (in 1 µl DMF) was added to 0.5 ml PRP from untreated rats 1 minute prior to addition of Arachidonic acid (1.5 mM). The concentration of TxB_2 was determined at maximal aggregation by RIA. N = 3-4.

[b]Significantly different from DMF.

NA = Not applicable.

1. Ibuprofen

The purpose of this study was to determine the effectiveness of ibuprofen to alter the response to MCTP. Rats were treated wtih MCTP (4 mg/kg) on Day 0, and were then treated with ibuprofen (10 or 17.5 mg/kg) or its saline vehicle by gavage 3 times daily through the end of the study. Rats were killed 14 days later, and lung injury and RVE were assessed. A preliminary study was performed to determine a dose of ibuprofen which would inhibit Tx synthesis and yet would not retard body weight gain. The drug effect was also confirmed in MCTP-treated rats.

a. Confirmation of drug effect. In a preliminary study to determine a dosing regimen which would effectively inhibit platelet aggregation and Tx synthesis, rats were treated with ibuprofen (IBN; 10 mg/kg orally, 3X daily) for 4 days. Blood was collected and PRP was prepared according to the second technique described in Methods. Platelet aggregation was induced with various concentrations of AA, and maximal aggregation was observed. The concentration of TxB_2 was also determined in PRP prior to aggregation.

The concentration of TxB_2 in PRP from rats treated with ibuprofen was significantly lower (0.4±0.1 ng/ml) than that from controls (1.2±0.2 ng/ml). The platelet aggregation response to a low concentration (0.6 mM) of AA was abolished in PRP from rats treated with IBN (Table 30). In contrast to the response in saline control rats, the platelet aggregation response in PRP from rats treated with IBN was dose-dependent, and the inhibition due to IBN treatment was overcome at higher doses of AA. On the basis of these results, doses of 10 and 17.5 mg/kg (orally, 3 times daily) were chosen for co-treatment of MCTP-treated rats.

Co-treatment of MCTP-treated rats with either dose of IBN reduced the concentration of circulating plasma TxB_2: plasma TxB_2

TABLE 30

Effect of Treatment with Ibuprofen (IBN)[a] In Vivo on Platelet Aggregation In Vitro

Concentration of Na^+-Arachidonate (mM)	% Maximal Aggregation	
	SAL	IBN
0.6	74±2	0±0[b]
0.9	73±2	38±15
1.5	71±2	72±7

[a]Rats received IBN (10 mg/kg) or saline orally 3 times daily for 4 days. Platelet aggregation responses in PRP were observed as described in METHODS. N = 4-6.

[b]Significantly different from SAL control ($p < 0.05$, rank sums test).

concentrations were 126±90 pg/ml in controls, and 30±20 and 27±8 pg/ml in rats treated with 10 and 17.5 mg/kg IBN, respectively (N = 4-6). These differences did not attain statistical significance due to the large variability in control values.

b. Effect of ibuprofen on MCTP-induced cardiopulmonary toxicity. Co-treatment with ibuprofen did not affect body weight in MCTP-treated rats. By comparison of the values in Table 31 to DMF controls at day 14 in Table 27, it can be seen that administration of MCTP resulted in elevation of lung weight and RVE. Ibuprofen, at either of the doses employed, was ineffective at reducing the MCTP-induced toxicity: lung weight/body weight and RV/(LV+S) in either ibuprofen group was not significantly different from the saline control group. The results of this study indicate that co-treatment with the cyclooxygenase inhibitor ibuprofen does not attenuate the response to MCTP.

2. Dazmegrel

Inhibition of cyclooxygenase can decrease synthesis of PGI_2 as well as TxA_2, and the vasodilatory and antiaggregatory effects of PGI_2 may be beneficial in MCTP-induced pulmonary hypertension. Therefore, a drug aimed more specifically at inhibiting TxA_2 biosynthesis was employed. The ability of the Tx synthetase inhibitor Dazmegrel to alter the cardiopulmonary response in MCTP-treated rats was examined at the onset (day 7) of pulmonary hypertension and after pulmonary hypertension was well-established (day 14). Treatment with Dazmegrel (50 mg/kg by gavage twice daily) or its saline vehicle began at the time of administration of DMF or MCTP (day 0, 3.5 mg/kg) and continued through the end of the study. Lung injury was assessed at day 7 and day 14, and RVE was assessed at day 14. A preliminary study was performed to determine an appropriate and effective dosing regimen.

TABLE 31

Lack of Effect of Ibuprofen (IBN) on the Cardiopulmonary Toxicity of MCTP[a]

Cotreatment	Saline	IBN (mg/kg)	
		10	17.5
$BW_{initial}$ (g)	229± 3	225±1	236± 3
BW_{final} (g)	241±17	234±3	268±10
WL/BW ($x10^3$)	8.6±1.1	7.3±0.9	8.1±0.6
RV/(LV+S)	0.34±0.02	0.30±0.02	0.35±0.02

[a]Rats were treated with IBN or with saline three times daily for 14 days following a single injection of MCTP (4.0 mg/kg, i.v.). BW = body weight; WL = wet lung weight. There were no significant differences among any of the means (one-way ANOVA, $p < 0.05$). N = 4-7.

a. Confirmation of drug effect

In a preliminary experiment to determine a dosing regimen which would effectively inhibit Tx synthesis, a small number (n = 2-3) of rats were treated with Dazmegrel or saline and were killed 3, 8, or 24 hours later. Blood was collected and PRP was prepared by the second technique described in Methods. The concentration of TxB_2 was determined in PPP and in PRP stimulated with AA (0.9 mM). The concentration of 6-keto $PGF_{1\alpha}$ was also determined in PPP. Three doses of Dazmegrel were tested: 25, 50, and 100 mg/kg.

The concentration of 6-keto $PGF_{1\alpha}$ in PPP was not affected by Dazmegrel treatment (Figure 30C). The TxB_2 in PRP and PPP 3 and 8 hours after treatment was reduced by all three doses of Dazmegrel (Figures 30A and B). By 24 hours the inhibitory effect disappeared. Both 50 and 100 mg/kg appeared to be slightly more effective at reducing the concentration of TxB_2 than 25 mg/kg, and on the basis of these data a dose of 50 mg/kg given twice daily was chosen for treatment of MCTP-treated rats.

Co-treatment with Dazmegrel decreased the concentration of TxB_2 in the plasma of rats treated 14 days earlier with MCTP or DMF, but it did not affect the plasma concentration of 6-keto $PGF_{1\alpha}$ (Table 32).

b. Effect of Dazmegrel on MCTP-induced cardiopulmonary toxicity. Co-treatment with Dazmegrel did not alter any of the indices of lung injury examined 7 days after treatment with MCTP (Table 33). Wet lung weight, lavage LDH activity and lavage protein concentration were elevated in MCTP-treated rats, and this increase was not affected by co-treatment with Dazmegrel. The wet/dry lung weight ratio was increased and body weight was decreased at day 7 in MCTP-treated rats co-treated with Dazmegrel.

Figure 30. The concentration of TxB_2 in (A) PRP and (B) PPP and (C) the concentration of 6-keto $PGF_{1\alpha}$ in PPP of rats treated 3 (top panel), 8 (middle panel), or 24 (bottom panel) hours earlier with Dazmegrel (DAZ) or the saline vehicle. PRP was prepared as described and was induced to aggregate with arachidonic acid (0.9 mM). Prostanoid concentration was determined by RIA. N = 2-3.

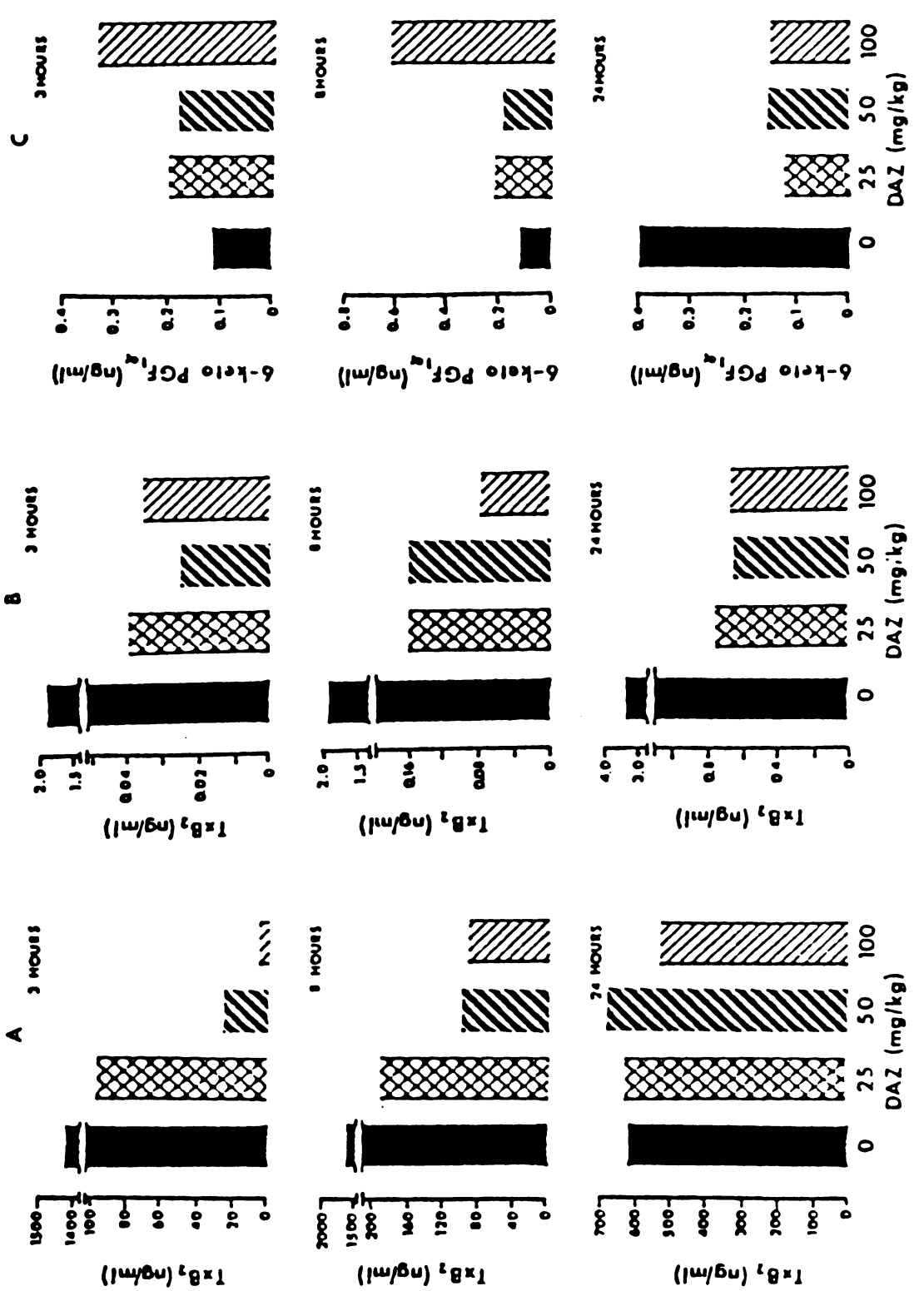
A
B
C
3 HOURS
8 HOURS
24 HOURS
TxB2 (ng/ml)
6-keto PGF1α (ng/ml)
DAZ (mg/kg)
0
25
50
100

Figure 30

TABLE 32

Plasma TxB_2 and 6-Keto $PGF_{1\alpha}$ in MCTP-treated Rats Following Co-treatment with Dazmegrel (DAZ)

	Treatment[a]			
	DMF		MCTP	
	SAL	DAZ	SAL	DAZ
TxB_2 (pg/ml)	290±190	14± 5[b]	250±110	27±10[c]
6-Keto $PGF_{1\alpha}$ (pg/ml)	230± 34	150±43	203± 36	279±73

[a]Rats received DAZ (50 mg/kg) or saline (SAL) orally, twice daily, for 14 days following a single injection of MCTP (3.5 mg/kg) or DMF, iv. TxB_2 and 6-keto $PGF_{1\alpha}$ were determined by RIA as described in METHODS. N = 6-9.

[b]Significantly different from DMF/SAL.

[c]Significantly different from MCTP/SAL.

TABLE 33

Lack of Effect of Dazmegrel (DAZ) on the Toxicity of MCTP
7 Days After Treatment

	Treatment[a]			
	DMF		MCTP	
	SAL	DAZ	SAL	DAZ
$BW_{initial}$ (g)	281±4	279±5	275±3	275±3
BW_{final} (g)	324±5	332±7	304±8	302±6[c]
WL/BW (x1000)	3.7±0.2	3.8±0.1	7.3±0.8[b]	7.8±1.1[c]
WL/DL	5.0±0.1	5.2±0.2	6.6±0.5	7.1±0.7[c]
Lavage LDH Activity (U/dl)	2.1±0.3	2.0±0.2	22.3±3.4[b]	18.3±3.3[c]
Lavage Protein Concentration (mg/ml)	0.13±0.02	0.13±0.02	2.46±0.59[b]	1.24±0.45[c]

[a]Rats were treated with MCTP (3.5 mg/kg) or DMF on day 0 and were co-treated with DAZ (50 mg/kg, orally, twice daily) or SAL. Animals were killed 7 days later. BW = body weight; WL = wet lung weight; DL = dry lung weight. N = 6-10.

[b]Significantly different from DMF/SAL.

[c]Significantly different from DMF/DAZ.

Body weight in rats treated 14 days earlier with MCTP was not affected by co-treatment with Dazmegrel (Table 34). Administration of MCTP resulted in an increase in wet lung weight and vascular leak, and these indices of lung injury were not affected by co-treatment with Dazmegrel. Similarly, Dazmegrel did not attenuate the elevation in lavage fluid protein concentration or LDH activity caused by MCTP. The wet/dry lung weight ratio was only elevated in MCTP-treated rats co-treated with Dazmegrel. Additionally, at day 14, RVE was not attenuated by co-treatment with Dazmegrel (Figure 31).

These results indicate that co-treatment with a thromboxane synthetase inhibitor does not afford protection from the toxicity of MCTP.

3. L-640,035

There is evidence that PGH_2, the precursor to TxA_2, can stimulate Tx receptors in vitro (Hamberg et al., 1974; Kadowitz et al., 1977). If this occurs in vivo, then treatment with a thromboxane synthetase inhibitor may not be sufficient to eliminate thromboxane receptor-mediated activities. Therefore, this study was undertaken to investigate whether co-treatment with a Tx receptor antagonist (L-640,035) could attenuate the pulmonary hypertensive response to MCTP. Rats were treated with MCTP (3.5 mg/kg) or DMF on day 0, and were co-treated by gavage with either L-640,035 (50 mg/kg) or its vehicle, polyethylene glycol (approximate molecular weight = 200, diluted with an equal volume of distilled water), three times daily. Treatment with L-640,035 started on day 0 and continued through day 14, when the animals were killed. Lung injury and pulmonary arterial pressure were assessed. As in previous drug treatment experiments, a preliminary study was performed to determine an effective dose.

TABLE 34

Lack of Effect of Dazmegrel (DAZ) on the Toxicity of MCTP 14 Days After Treatment

	Treatment[a]			
	DMF		MCTP	
	SAL	DAZ	SAL	DAZ
$BW_{initial}$ (g)	275±4	272±6	278±3	278± 3
BW_{final} (g)	336±5	337±7	300±8	286±12
WL/BW (x 10^3)	3.6±0.1	3.6±0.1	6.7±0.6[b]	9.4±1.1[c]
WL/DL	5.5±0.6	5.0±0.2	6.1±0.1	7.0±0.4[c]
Lavage LDH activity (U/dl)	2.5±0.2	2.2±0.2	13.7±2.3[b]	13.8±1.9[c]
Lavage protein concentration (mg/ml)	0.16±0.02	0.20±0.03	2.89±0.28[b]	2.85±0.55[c]
^{125}I-Lung/^{125}I-blood	0.22±0.03	0.26±0.03	0.64±0.10[b]	0.87±0.13[c]

[a]Rats received DAZ (50 mg/kg, orally, twice daily) or SAL for 14 days following treatment with MCTP (3.5 mg/kg) or DMF. BW = body weight; WL = wet lung weight; DL = dry lung weight. N = 6-17.

[b]Significantly different from DMF/SAL.

[c]Significantly different from DMF/DAZ.

Figure 31. Right ventricular enlargement in MCTP-treated rats following co-treatment with Dazmegrel (DAZ). Rats were treated as described in Table 34 and were killed 14 days later. N = 12-16. a = significantly different from DMF/SAL. b = significantly different from DMF/DAZ.

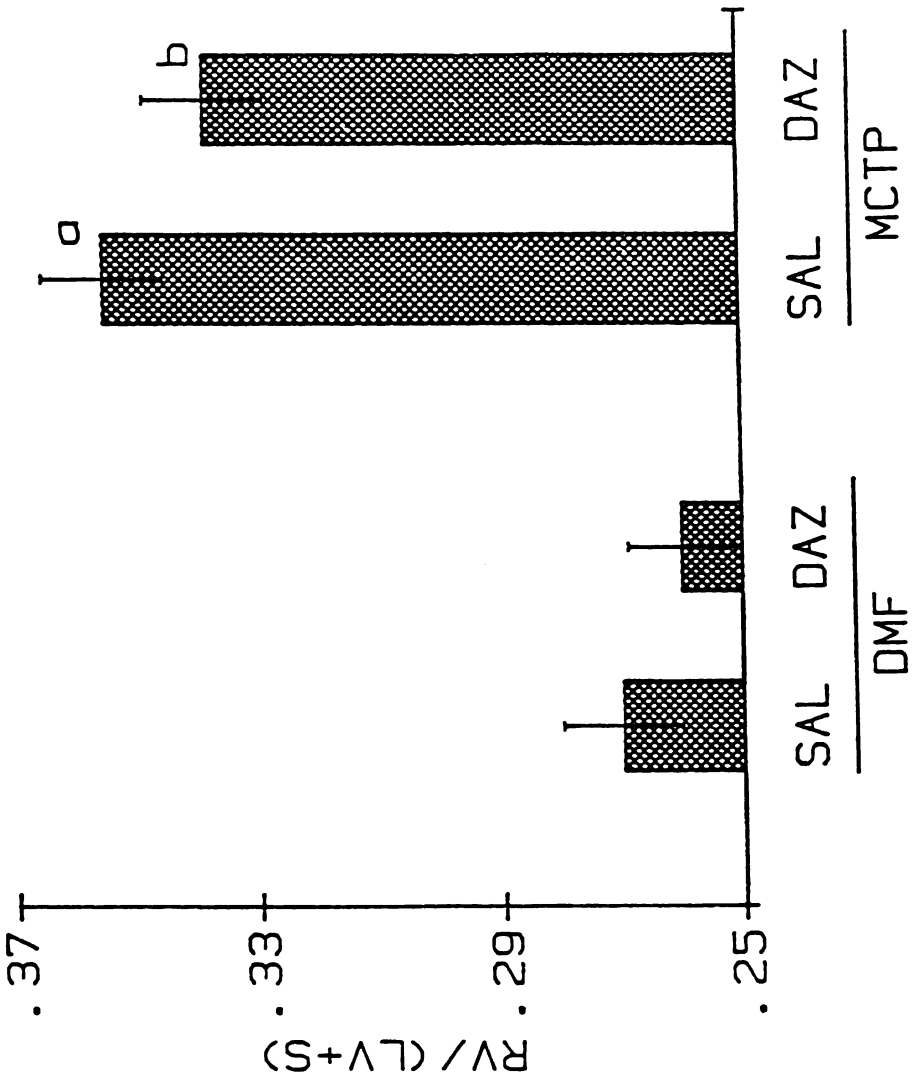

Figure 31

a. Confirmation of drug effect

In a preliminary study to determine a dosing regimen of L-640,035 which would antagonize vascular Tx receptors, rats not treated with MCTP were given a single oral dose of L-640,035 (50 mg/kg) or its vehicle. Two or eight hours later the increase in mean right ventricular pressure induced by the thromboxane mimic U46619 was measured.

Baseline right ventricular pressure was slightly reduced in L-640,035-treated rats (control, 14.2±0.8 mmHg; L-640,035-treated, 10.7±0.9 and 9.7±1.7 mmHg at 2 and 8 hours, respectively; N = 3-6), and this difference was statistically significant at 8 hours. The increase in right ventricular pressure in response to U46619 was dose-dependent in treated and control rats (Figure 32). Two or eight hours after treatment, the right ventricular pressure response to the same dose of U46619 was less in L-640,035-treated animals than controls. The response to the intermediary dose of U46619 (1.5 μg/kg) was depressed by 73% two hours after treatment and by 59% eight hours after treatment. Based on these results, a dosing regimen of 50 mg/kg, 3 times daily was chosen.

The effectiveness of L-640,035 in MCTP-treated rats was confirmed by its ability to antagonize the right ventricular pressure response to the thromboxane mimic. This response was depressed by 63% in MCTP-treated rats by co-treatment with L-640,035 (Table 35).

b. Effect of L-640,035 on MCTP-induced cardiopulmonary toxicity. Body weight gain was less in MCTP-treated rats co-treated with L-640,035 (Table 36). Administration of MCTP resulted in increases in lung weight and in lavage fluid protein concentration which were unaffected by co-treatment with L-640,035. Lavage fluid LDH activity was elevated in both groups of MCTP-treated rats, however, this only reached statistical significance in rats co-treated with L-640,035. The wet/dry lung weight ratio was not affected by treatment

Figure 32. Effect of treatment with L-640,035 on the dose/response relationship to U46619. Rats were treated with the vehicle (open squares) or with L-640,035 (50 mg/kg, orally) 2 (closed circles) or 8 (closed diamonds) hours earlier. Mean right ventricular pressure (RVP) following intravenous injection of U46619 was measured as described in METHODS. Pressure responses in vehicle-treated rats at 2 and 8 hours were not different, and were pooled for comparison. At the two lower doses of U46619, values for rats treated 2 or 8 hours earlier are significantly different from vehicle controls (one-way ANOVA, $p < 0.05$). N = 3-6.

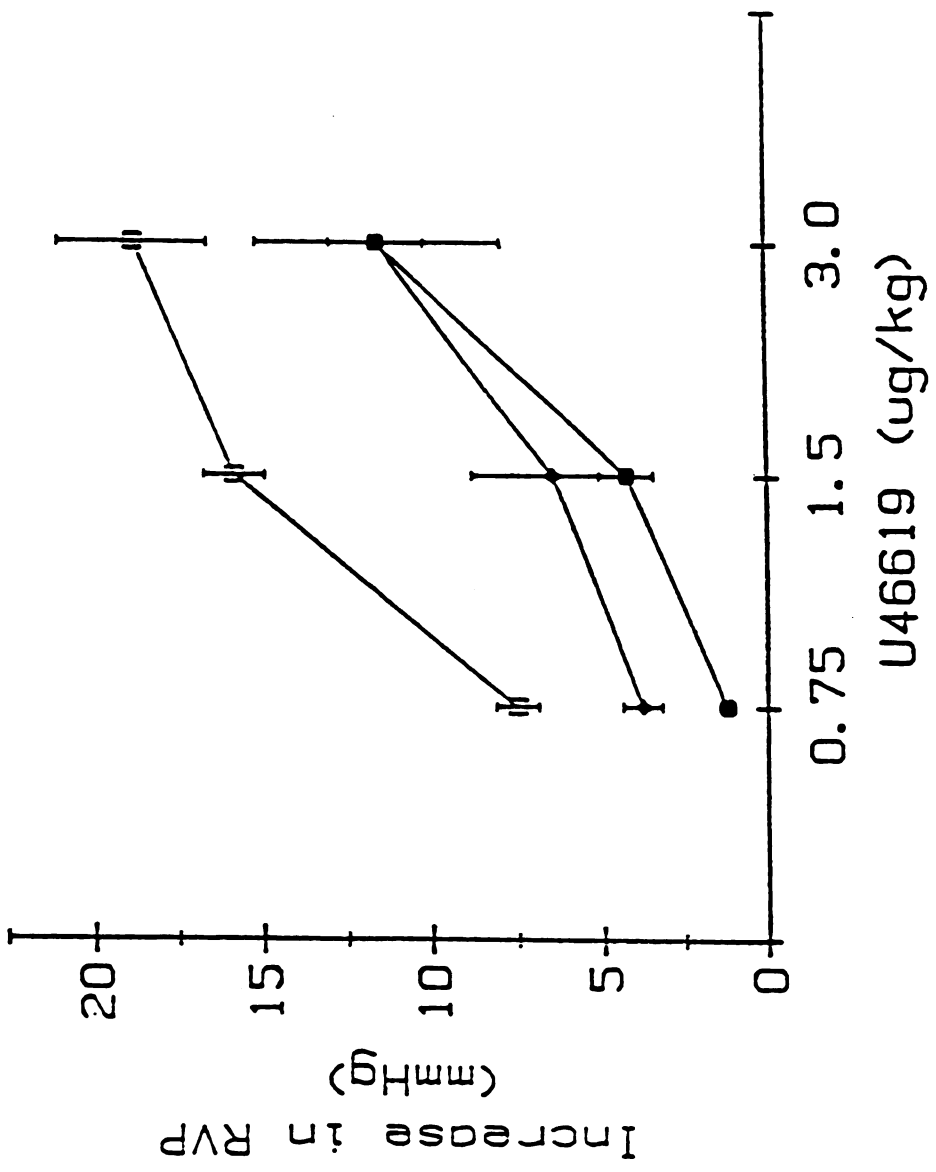

Figure 32

TABLE 35

Right Ventricular Pressure Response to U46619 in MCTP-treated Rats Following Co-treatment with L-640,035

	Treatment[a]			
	DMF		MCTP	
	VEH	L-640,035	VEH	L-640,035
RVP baseline (mmHg)	12.1±1.2	12.5±0.4	21.2±2.2[b]	19.7±2.7[c]
Increase in RVP in response to U46619 (mmHg)	6.8±1.6	3.8±0.6	13.9±1.3[b]	5.1±0.8[d]

[a]Rats were treated with L-640,035 (50 mg/kg) or its vehicle (VEH) orally 3 times daily. 14 days after MCTP (3.5 mg/kg) administration, mean right ventricular pressure (RVP) was recorded before, and in response to, an intravenous injection of U46619 (1.5 μg/kg). N = 3-5.

[b]Significantly different from DMF/VEH.

[c]Significantly different from DMF/L-640,035.

[d]Significantly different from MCTP/VEH.

TABLE 36

Lack of Effect of Co-treatment with L-640,035 on MCTP-induced Toxicity

	Treatment[a]			
	DMF		MCTP	
	VEH	L-640,035	VEH	L-640,035
$BW_{initial}$ (g)	230±4	228±4	233±6	228± 6
BW_{final} (g)	310±3	308±9	287±8	249±17[c]
WL/BW (x1000)	3.3±0.2	3.5±0.1	6.3±1.0[b]	6.9±1.3[c]
WL/DL	4.5±0.3	5.1±0.2	5.9±0.5	6.2±0.4
Lavage LDH activity (U/dl)	2.2±0.2	2.7±0.2	5.4±1.4	10.9±3.1[c]
Lavage protein concentration (mg/ml)	0.13±0.01	0.14±0.01	1.5±0.4[b]	2.2±0.7[c]

[a]Rats were treated with L-640,035 (50 mg/kg, p.o., 3 times daily) or its vehicle (VEH) following treatment with MCTP (3.5 mg/kg). BW = body weight; WL = wet lung weight; DL = dry lung weight. N = 4-6.

[b]Significantly different from DMF/VEH.

[c]Significantly different from DMF/L-640,035.

with MCTP or with L-640,035. MCTP treatment caused elevations in pulmonary arterial pressure (Figure 33) and right ventricular pressure (Table 35) which were unaffected by co-treatment with L-640,035.

These results indicate that co-treatment with a thromboxane receptor antagonist does not attenuate the pulmonary hypertensive response to MCTP.

Figure 33. Mean pulmonary arterial pressure (PAP) in MCTP-treated rats following co-treatment with L-640,035 (L640). Rats were treated with MCTP (3.5 mg/kg) or DMF and were co-treated with L-640,035 (50 mg/kg, p.o., 3 times daily) or its vehicle (VEH). PAP was measured 14 days after treatment with MCTP. N = 4-5. a = significantly different from DMF/VEH. b = significantly different from DMF/L-640,035.

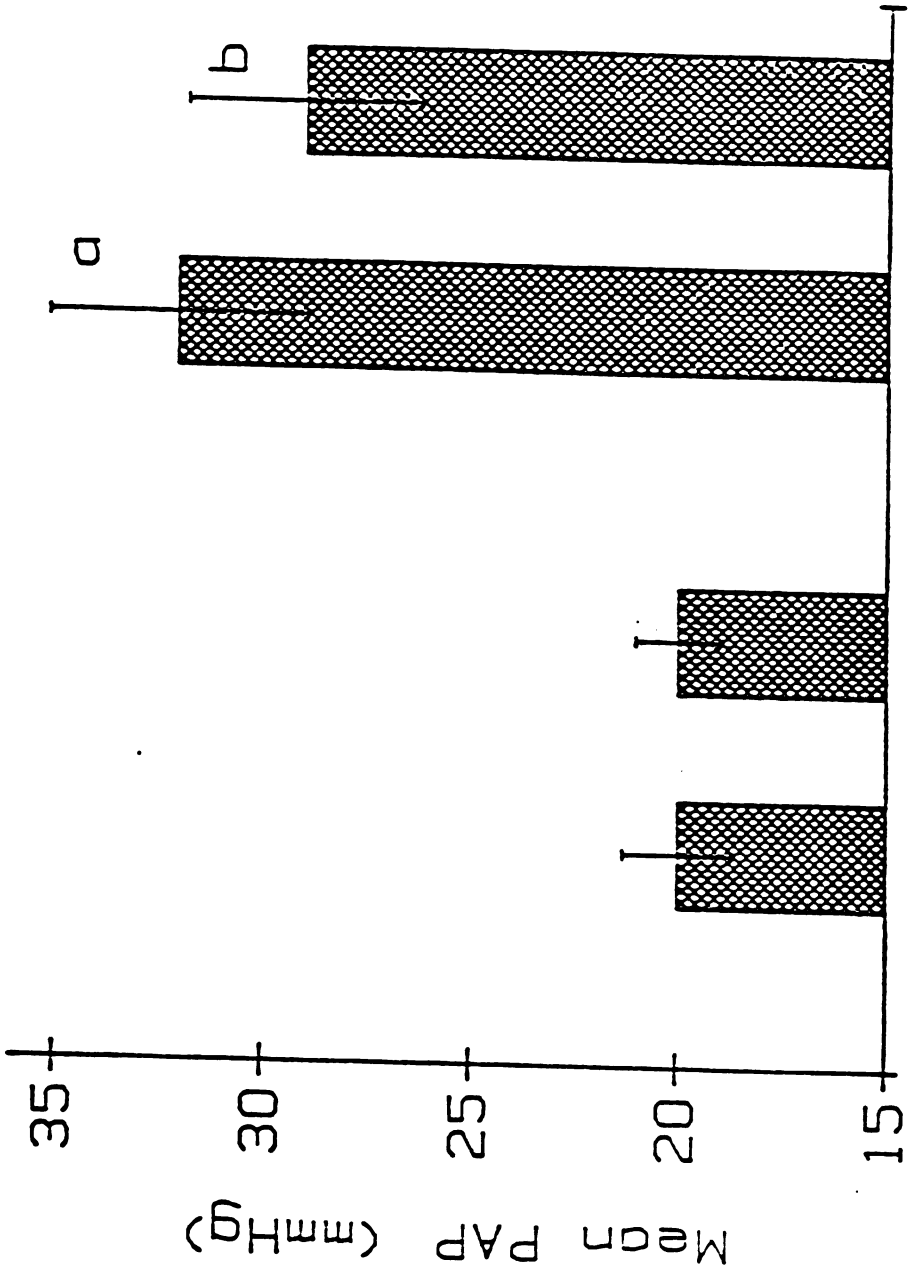

Figure 33

DISCUSSION

I. The Dose/Response Relation for MCTP

This study was undertaken to determine a dose of MCTP which would produce right ventricular enlargement (RVE) by day 14 after treatment and yet would not cause significant mortality. Results from previous studies indicated that RVE was apparent at day 14 following treatment with 5 mg MCTP/kg (Bruner et al., 1983), however, in subsequent studies as many as 50% of animals treated with this dose of MCTP died within that time period. This raised the concern that animals which survived through the end of the study may represent a resistant population. Chesney and coworkers (1974a) demonstrated that MCTP at doses of 2 and 4 mg/kg produced lung injury and RVE by 4 weeks after treatment. Therefore, in this study, doses of MCTP ranging from 2 to 5 mg/kg were employed.

By day 14, lung injury and RVE were apparent at all doses of MCTP (Figure 5), while deaths were only observed at the highest dose (5 mg/kg) of MCTP. These results indicate that, at doses between 2 and 4 mg/kg, MCTP produces RVE (and presumably pulmonary hypertension) without causing substantial mortality. Based on these results, in subsequent studies MCTP was given at doses ranging from 3.5 to 5 mg/kg.

II. The Development of MCTP-Induced Toxicity

Although there have been numerous studies describing the pathology associated with MCT treatment, fewer have been reported for MCTP, and none of these

have attempted to correlate biochemical and physiological alterations with changes observed at the light microscope level. For this purpose, the development of MCTP-induced pneumotoxicity was assessed by both biochemical methods and histological examination.

The earliest time after MCTP treatment examined was three days. This time was chosen because in previous studies no biochemical or physiological indices of lung injury were observed at day 3 (Bruner et al., 1983), and it was of interest to determine if subtle changes were occurring at this time that could only be detected by histologic examination. In contrast to previous results (Bruner et al., 1983, 1986), cell injury, assessed as an increase in lavage fluid LDH activity, and vascular leak was evident 3 days after treatment with MCTP (Table 3). The magnitudes of these increases, though, were not as great as were observed at later times after treatment. Consistent with increased vessel leak, perivascular edema was noted histologically. Although the perivascular edema was observed predominantly around medium and large muscular vessels, it was not clear in which vessels increased leak was occurring. Perivascular edema was associated with dilation of lymphatics in the interstitium so that it is possible that vessel leak was occurring in capillaries, increasing lymph drainage to the larger vessels, and causing perivascular edema in these vessels. Alternatively, fluid escaped from larger vessels could have contributed to perivascular edema noted there. The mild cellular infiltrate observed may have increased the dry weight of the lung, resulting in a decreased wet/dry lung weight ratio at this time.

Although endothelial cell (EC) changes have been reported 24 hours after treatment with MCT (Valdivia et al., 1967), in the present study, 3 days after MCTP no intimal alterations of vessels were observed by light microscopy. This is consistent with the findings of Butler and coworkers (1970), who observed no changes in the ECs two days after administration of MCTP, but did observe

changes one week after treatment. Tissues in the present study were only examined at the light microscope level, and perhaps examination by electron microscopy would have revealed subtle changes in ECs not detectable by light microscopy. The pulmonary EC has been considered to be the target of MCT and MCTP toxicity, as metabolites of MCT or MCTP arrive in the lung via the blood and ECs are the first pulmonary cells encountered. However, evidence for remarkable alterations in ECs shortly after treatment with MCTP is limited. Certainly in this study pulmonary vascular alterations observed histologically at day 3 were unimpressive, even though some evidence of minor pulmonary injury was already apparent.

Five days after administration of MCTP perivascular edema was more severe and was associated with smaller sized vessels. This correlated with an increase in wet lung weight, in the wet/dry lung weight ratio, and in vascular leak. Cell injury was evidenced by an increase in lavage fluid protein concentration and LDH activity, and this was accompanied by increased margination of WBCs and an infiltration into the perivascular connective tissue of hypertrophic lymphomononuclear cells and occasional mast cells. As at day 3, vascular lesions were largely unimpressive, and the more pronounced changes occurred in the interstitium and the airways. Alveolar septae were thick with edema and infiltrated cells, alveolar Type II cells were hypertrophic, and enlarged macrophages were common in alveolar lumens. This suggested that at day 5 the major response to injury was occurring in the airways and interstitial spaces, not the vasculature.

By day 8 a generalized proliferative response of many cell types was seen in lungs of treated rats, and nuclear dust and increased numbers of mitotic figures were observed. Consistent with previous reports for MCT (Kay et al., 1982a; Sugita et al., 1983a) and MCTP (Butler et al., 1970), Type II pneumocytes and

alveolar interstitial cells were hypertrophic. Clara cell blebbing was less pronounced, suggesting that these cells may be differentiating to replace injured cells of the bronchiolar epithelium. In more severely affected areas, hypertrophy of ECs and of smooth muscle cell nuclei was observed. The marked cellular proliferation might suggest the presence or activation of some growth factor or mitogen in response to MCTP.

Edema fluid was observed in the perivascular interstitium, alveolar septal walls, and alveolar lumens, and was accompanied by an infiltration of lymphomononuclear cells and large foamy alveolar macrophages. This accumulation of fluid resulted in increased wet lung weight and was consistent with increased vascular leak. The wet/dry lung weight ratio was not different from control, perhaps reflecting an increase in both fluid accumulation and cellularity.

At day 8 margination of WBCs increased due to treatment, and platelet thrombi were occasionally observed. Alterations of vascular smooth muscle were confined to separation of smooth muscle cells due to edema fluid and occasional hypertrophy of smooth muscle cell nuclei, and were observed in 2 of 3 treated rats with elevated PAP and in 1 of 2 treated rats with normal PAP. Detailed morphometric analysis of vessel size and medial thickness was not performed; however, it did not appear that smaller pulmonary vessels of MCTP-treated rats were thicker than those of controls. Perhaps minor changes in medial thickness would become apparent if detailed morphometry was performed. In seeming contrast to many pathology studies performed with MCT, 8 days after treatment with MCTP vascular alterations were not severe, intimal changes were minor, and the most prominent changes were observed in the airways and interstitial tissue.

Two weeks after administration of MCTP, biochemical and histological evidence of severe lung injury was observed. Increases in wet lung weight, wet/dry lung weight ratio, and vascular leak correlated with severe and extensive

perivascular and alveolar edema. Marked cell injury accompanied increases in lavage protein concentration and LDH activity. As has been previously reported (Butler et al., 1970; Plestina and Stoner, 1972), numerous alterations in bronchiolar and alveolar epithelial cells were also observed. Also consistent with a previous report (Turner and Lalich, 1965), the bronchiolar epithelium extended to alveolar ducts and sacs, an alteration which could impair gas exchange.

At day 14, pulmonary hypertension and RVE were apparent. At this time, EC injury was a frequent finding, as were separation and hypertrophy of medial smooth muscle cells of pulmonary vessels. In addition, some vessel walls appeared thickened, although this was not confirmed by morphometric analysis.

To summarize the development of MCTP-induced toxicity, following a single intravenous injection of MCTP, biochemical and physiological indices of lung injury as well as lesions identified histopathologically increase in number, extent, and severity with time. The first indices of injury observed were increases in lavage LDH activity and vascular leak at day 3. This was accompanied by perivascular edema and a mild infiltration of lymphomononuclear cells. Two days later, lung injury had progressed. By day 8, three of five treated rats had elevated PAPs, although vascular lesions were not prominent. The most pronounced alteration was the proliferation of many cell types in the airways and interstitium. It was not until 14 days after treatment that vascular lesions, including alterations of ECs and smooth muscle cells, were more pronounced, although still not the most prominent change observed. At this time pulmonary hypertension was evident in all treated rats.

A few observations resulting from this study differ from those reported previously for MCT or MCTP. EC changes were noted by electron microscopy within 24 hours (Valdivia et al., 1967) and by light microscopy (Turner and Lalich, 1965) and electron microscopy (Merkow and Kleinerman, 1966) somewhat later

after treatment with MCT. EC changes were also noted by electron microscopy within one week after treatment with MCTP (Butler et al., 1970). Only minor alterations in ECs were observed in this study, and not until day 14 after treatment with MCTP. Likewise, marked vascular smooth muscle changes and medial hypertrophy were not observed in this study, in contrast to previous reports for MCT (Turner and Lalich, 1965; Ghodsi and Will, 1981; Kay et al., 1982a) and MCTP (Chesney et al., 1974a). The most impressive vascular changes observed in this study occurred in the adventitia, and the most pronounced alterations in the pulmonary tissue were observed in the alveolar septae and spaces, and in the perivascular interstitium. However, minor injury was observed in the pulmonary vasculature, and more subtle lesions may have been present but not detectable by light microscopy. Conceivably, more subtle vascular injury could cause a chronic, low-level release of mediators from platelets that may promote injury and pulmonary hypertension.

In retrospect, it would have been of interest to examine a time earlier than day 3, before any injury was apparent (as had been planned), and to examine a time between days 8 and 14 when all rats had elevated pulmonary arterial pressure but when the alterations in the lung were less severe than at day 14. Although detailed morphometry is not available at this time, it appears as though pulmonary hypertension developed in some rats at day 8 before any remarkable medial thickening or smooth muscle extension was apparent. This may suggest a role for vasoconstriction in MCTP-induced pulmonary hypertension. Vessel leak and the resulting edema were also apparent well before any elevations in pulmonary arterial pressure were observed, so that vessel leak may also contribute to MCTP-induced pulmonary hypertension.

III. Influence of Diet Restriction on MCTP-Induced Cardiopulmonary Toxicity

The observation that diet reduction attenuated the cardiopulmonary toxicity of MCT (Hayashi et al., 1979) prompted an investigation to determine whether this protection was due to an effect of decreased bioactivation of MCT induced by diet restriction. In rats treated 14 days earlier with MCTP, which does not require bioactivation (Bruner et al., 1986), restriction of food intake reduced the MCTP-induced elevation of lung weight and lavage fluid protein concentration and prevented the development of RVE (Figure 7). The elevation in lavage LDH activity caused by MCTP was not affected by diet restriction, however. This lack of effect on LDH activity may be explained by the observation that lavage fluid LDH activity is maximal 10 days following a single injection of MCTP, but by 14 days returns rapidly toward control values (Bruner et al., 1983). Due to this rapid change, the variation in lavage LDH values in MCTP-treated rats is large at this time. Although there was a trend toward decreased LDH activity in lavage fluid of diet-restricted rats (Figure 7), this did not attain statistical significance.

These results indicate that reduction of food intake attenuates the toxicity of MCTP, and they suggest that the protective effect of diet restriction seen in MCT-treated rats was not due to decreased bioactivation of MCT. The mechanism by which diet restriction lessens effects produced by MCTP remains unknown. Alterations in intake of specific dietary components produce a variety of physiological and biochemical changes. For example, dietary fat content can affect platelet function in a number of ways. Certain variations in dietary fat composition have been shown to increase thrombin-induced platelet aggregation and to decrease aggregation responses to ADP (McGregor and Renaud, 1978). A change in dietary fat has also been associated with an increased concentration of cyclic AMP in platelets, which would inhibit platelet aggregation, and with increased serum concentrations of PGE_1 (Fine et al., 1981), which downregulates

platelets. Furthermore, alterations in dietary fat can also affect the pulmonary vasculature: isolated lungs and isolated pulmonary vessels of rats raised on an essential fatty acid-deficient diet are decreased in responsiveness to some vasoconstrictive stimuli (Morganroth et al., 1985).

Another possible explanation for the observed protective effects of dietary restriction relates to the suppression of weight gain. MCTP-treated rats eating ad lib did not gain weight, and treated rats restricted to 9 g food/rat/day lost weight over the two-week period following treatment (Figure 6). Hayashi and coworkers (1979) reported that in MCT-treated rats pulmonary alterations progress in association with increases in body weight. It is possible that some of the MCT- or MCTP-induced cardiopulmonary responses (for example, RVE) require nutrition adequate for growth, so that toxicity is attenuated in animals which cannot grow. Along these lines, it has been reported recently that dietary reduction attenuates the increase in polyamines and their biosynthetic enzymes in lungs of MCT-treated rats (Hacker and Isaghulian, 1986). Since polyamines are thought to be necessary for cell growth and proliferation, and since dietary reduction inhibits polyamine biosynthesis, this may suggest that reduction of food intake attenuates MCTP-induced toxicity by decreasing production of the necessary requirements for cell growth and proliferation.

There was a trend toward prolonged survival of diet-restricted, MCTP-treated rats as compared to those eating ad lib; however, beyond 30 days following treatment there were no significant differences in the number of animals surviving in the two groups (Figure 8). It was noticed that ad lib animals died more quickly once they became sickly in appearance, while diet-restricted animals seemed to survive in this weakened condition for a longer time. It is not known whether MCTP-treated animals die from pulmonary complications or right heart failure. In this study, the post mortem RV/(LV+S) determined in animals

which did not survive 40 days was not significantly different in ad lib and diet-restricted animals (data not shown). However, among the surviving animals, the diet-restricted group had a lower RV/(LV+S) than animals allowed to eat ad lib (Table 6). This could indicate that animals which develop RVE despite reduction in food intake may not be protected from MCTP-induced lethality. However, the significance of RV/(LV+S) measured after death has not been established, and this observation should be interpreted with caution.

The modest effect of diet restriction on survival after MCTP treatment differed in degree from the marked protection observed after MCT administration (Hayashi et al., 1979). No mortality was observed in MCT-treated rats on reduced food intake for 90 days. However, when MCT-treated rats which had been diet-restricted for 30 days were allowed free access to food again, they began to die after 10 days. This suggests that MCT may produce a condition which is not lethal until aggravated by food consumption, some specific dietary component, or growth.

In a separate series of experiments not presented here, the possibility that sodium was the specific dietary component responsible for the protective effect of diet restriction was tested (Ganey et al., 1985). Increasing or decreasing the sodium intake of MCTP-treated rats did not alter the cardiopulmonary response to MCTP. Therefore, the protective effects of diet restriction are not due to an alteration in sodium intake.

In summary, reduction of food intake attenuates the pulmonary toxicity of MCTP and prevents development of RVE. Diet-restricted animals survive for a longer time, but this protective effect is short-lived. The mechanism by which reduction of food intake attenuates the toxic response to MCTP is not known. However, many drugs given subacutely retard body weight gain in animals, so that the results of these studies indicate that changes in body weight and food

consumption must be considered when evaluating the effect of drug treatments on the toxicity of MCTP.

IV. The Effect of Thrombocytopenia on MCTP-Induced Pulmonary Hypertension

The observation that antibody-induced thrombocytopenia reduced the severity of RVE in MCTP-treated rats (Hilliker et al., 1984a) suggested that blood platelets may be involved in the pulmonary hypertensive response to MCTP. The observation that lung injury assessed 14 days after treatment with MCTP was not different in normal and thrombocytopenic rats might suggest that reducing the number of circulating platelets either delayed the toxic effects of MCTP or did not affect lung injury. One interpretation of the second possibility is that lung injury and pulmonary hypertension in this model are dissociable, and perhaps not causally related. This idea is not firmly supported in the literature: although there are a few reports of interventions which ameliorate the pulmonary hypertensive response and not lung injury, the converse has never been reported. Another interpretation is that lung injury initiates platelet involvement in the development of pulmonary hypertension. The purpose of the series of experiments performed here was to provide direct evidence that reducing the circulating platelet number attenuated MCTP-induced pulmonary hypertension at day 14, and to explore the possibility that thrombocytopenia delayed the development of MCTP toxicity.

To accomplish the first objective, direct measurement of pulmonary arterial pressure (PAP) was performed in thrombocytopenic rats 14 days after administration of MCTP. MCTP-treated rats with normal numbers of circulating platelets developed pulmonary hypertension and RVE, while in MCTP-treated rats with platelet numbers approximately 24% of control, this response was abolished (Figures 14 and 15). MCTP-induced lung injury was not altered by reduction of

platelet numbers (Tables 13 and 14). Thus, these results confirm the finding that thrombocytopenia protects against MCTP-induced RVE, and extend that effect to protection against elevation of pulmonary arterial pressure as well. This also supports the contention that RVE develops in response to pulmonary hypertension in this model.

This particular experiment did not address the possibility that the protective effect was observed at day 14 because thrombocytopenia delayed the onset of MCTP-induced toxicity. In rats with normal platelet numbers, increases in lavage LDH activity and vascular leak are first observed by day 3, and lung injury is evident with all indices we used by 5 days after administration of MCTP (Table 3). Pulmonary hypertension, however, does not develop until day 8 or later. Thus, if the onset of toxicity were delayed by as long as one week, lung injury would still be apparent by day 14, yet pulmonary hypertension would not have developed.

To test whether thrombocytopenia delayed MCTP-induced toxicity, lung injury and pulmonary hypertension were to be assessed in PAS-treated rats 8 days after administration of MCTP. However, in the course of these experiments it was discovered that the degree of thrombocytopenia achieved was critical for the protective effect. MCTP-treated rats in which platelet number was reduced below 10% of normal developed RVE and pulmonary hypertension by day 14 which was not different from MCTP-treated rats with normal numbers of platelets (Figures 12 and 13). The disparate effects of this severe thrombocytopenia and the more modest reduction of platelet numbers which prevented MCTP-induced pulmonary hypertension may be explained by some protective function of the platelet in the latter but not the former situation. That is, platelets may be necessary for some homeostatic mechanism, and it may be that circulating platelet numbers above 10% of normal are required for this function. For example, it is believed that platelets support vascular endothelium and maintain

vascular integrity by a mechanism separate from hemostasis or vascular repair (Gimbrone et al., 1969; Roy and Djerassi, 1972). In dogs made thrombocytopenic, vascular integrity was restored after infusion of far fewer platelets than were required to return bleeding time to normal (Roy and Djerassi, 1972). Human thrombocytopenia (Kitchens and Pendergast, 1986) and thrombocytopenia experimentally-induced in animals (Kitchens and Weiss, 1975) is associated with hemorrhage, vessel leak, and alterations in capillary endothelium, including thinning of the endothelial cell cytoplasm and the appearance of fenestrations. Although hemorrhage and vessel leak are consistent findings in thrombocytopenic animals, other studies have demonstrated that red blood cells leave the vessel lumen through normal endothelium (Van Horn and Johnson, 1966; Dale and Hurley, 1977; Shepro et al., 1980). In either case, the fact that vascular integrity is preserved at lower levels of circulating platelets than are other platelet functions may explain the different effects of severe and modest thrombocytopenia on MCTP-induced pulmonary hypertension. For example, it might be that in rats for which the degree of thrombocytopenia was severe (and not in rats with modest thrombocytopenia), vessel leak caused an increase in pulmonary arterial pressure which obscured any protective effect due to decreased numbers of platelets.

By what mechanism is thrombocytopenia affording protection against the pulmonary hypertension caused by MCTP? In experiments performed here and elsewhere (Figures 14 and 15 compared to Tables 11 and 12; Hilliker et al., 1984a), the response to MCTP was attenuated to the greatest extent when rats were thrombocytopenic from days 6-8. In rats with normal platelet numbers, 8 days after treatment with MCTP a generalized proliferative response is observed in the lung, and cell injury is severe. If cell injury and repair are related to the development of pulmonary hypertension, this might suggest that the platelet is providing a stimulus for cell growth or a mediator which injures cells. This would

fit well with the finding that inhibitors of polyamine biosynthesis attenuate the response to MCT (Olson et al., 1984b). The platelet contains and releases a number of growth factors which are mitogenic for smooth muscle (Deuel and Huang, 1984) and endothelial cells (King and Buchwald, 1984), and also releases TxA_2, which can cause injury to endothelial cells (DeClerck et al., 1985a).

Alternatively, the platelet may be providing a vasoconstrictive mediator, the actions of which are confined to the pulmonary vascular bed because that is the site of vascular injury and, therefore the site of platelet activation. Intravascular platelet aggregation and mediator release have been associated with increased pulmonary vascular pressure and pulmonary edema (Bo and Hognestad, 1972; Vaage et al., 1974; 1976). Two vasoconstrictive mediators released by platelets are 5-hydroxytryptamine and thromboxane A_2, and their roles in MCTP-induced pulmonary hypertension are addressed in the next section.

Another possible mechanism by which thrombocytopenia may afford protection is dependent not on the period of reduced platelet numbers, but on the reappearance and overshoot of platelets observed after treatment with the anti-platelet serum (Figure 16). When rats are made thrombocytopenic from days 6-8 after MCTP treatment, the overshoot of platelet number occurs from days 11-14. Platelets from MCTP-treated rats not co-treated with anti-platelet serum were hyporesponsive to aggregation by adenosine diphosphate, collagen, and arachidonic acid 14 days after MCTP (Hilliker et al., 1983b). If this decreased responsiveness is involved in MCTP-induced pulmonary hypertension, it is possible that the presence of newly-formed (and presumably normally-responsive) platelets, or the presence of a greater number of platelets, could be protective against pulmonary hypertension. The time course of these effects may argue against this possibility, though. In rats with normal numbers of platelets, pulmonary hypertension begins to develop by day 8 and is well established by day 14. Platelets respond normally

to most aggregating agents at day 7 but are hyporesponsive at day 14 (Hilliker et al., 1983b). It seems unlikely that an effect on platelets at day 14 may be a cause of the pulmonary hypertension which began 6-7 days earlier. Platelet numbers in thrombocytopenic rats do not increase above the numbers seen prior to the administration of the anti-platelet serum until day 11, a time when pulmonary hypertension already exists. Therefore, it seems more likely that the reduction in platelet number (and not the platelet rebound) is responsible for the protective effect observed in MCTP-treated rats made thrombocytopenic.

In summary, reduction of platelet number to approximately 20% of normal prevented MCTP-induced pulmonary hypertension and RVE observed at day 14. This effect was not observed when the degree of thrombocytopenia was more severe. It is not known by what mechanism thrombocytopenia protects against MCTP-induced pulmonary hypertension, but the platelet may be providing a vasoconstrictive mediator. This possibility is discussed in detail below.

V. The Role of Platelet Mediators in MCTP-induced Cardiopulmonary Toxicity

The platelet stores or synthesizes a number of vasoactive mediators which can be released during platelet activation. Two vasoconstrictors released by platelets, 5-hydroxytryptamine (5HT) and thromboxane A_2 (TxA_2), were considered as possible participants in MCTP-induced pulmonary hypertension.

A. 5-Hydroxytryptamine

It seemed reasonable that 5HT may be involved in MCTP-induced pulmonary hypertension for a number of reasons. First, it has been reported that endothelial cell damage is prominent in the pulmonary vessels of rats treated with MCT (Turner and Lalich, 1965; Merkow and Kleinerman, 1966; Valdivia et al., 1967) or MCTP (Butler, 1970; Chesney et al., 1974a). Relatively high doses of MCTP produce pulmonary edema within 18 hours (Hurley and Jago, 1975),

suggesting that MCTP is also capable of causing EC injury. Although no marked alterations in EC were observed by light microscopy after a relatively low dose of MCTP in the present study, more subtle vascular lesions may not have been detected. Platelets activated by interaction with injured vessels release 5HT which can cause vascular contraction of isolated vessels (Cohen et al., 1981; DeClerck and Van Nueten, 1982; Mullane et al., 1982; McGoon and Vanhoutte, 1984; Ogunbiyi and Eyre, 1984), can increase vascular pressure in isolated, perfused lungs (Figure 20), and can cause pulmonary hypertension in vivo (Breuer et al., 1985). Subtle vascular lesions may cause a low-level release of 5HT which could increase pulmonary vascular pressure chronically. In addition, 5HT can potentiate the response to aggregating agents (DeClerck et al., 1982a; Glusa and Markwardt, 1984) or to other vasoconstrictors (DeClerck and Van Nueten, 1982; Van Nueten et al., 1982). Therefore, elevated release of 5HT in the lungs of MCTP-treated rats could contribute to pulmonary hypertension.

Secondly, 5HT is removed from the pulmonary circulation by carrier-mediated uptake into endothelial cells, followed by intracellular inactivation by monoamine oxidase (Gillis and Pitt, 1982; Roth, 1985). Removal of 5HT is depressed in isolated, perfused lungs of rats following treatment with MCT or MCTP (Gillis et al., 1978; Hilliker et al., 1982, 1983c). This decreased inactivation of 5HT by the pulmonary endothelium could cause an elevated pulmonary concentration of this amine, and again, pulmonary vasoconstriction.

Platelets also actively accumulate 5HT and thereby remove free 5HT from the pulmonary circulation (Born et al., 1972; Steinberg and Das, 1980; Given and Longenecker, 1985), and decreased accumulation by platelets could thus result in an increased pulmonary concentration of free 5HT. It is unlikely that decreased accumulation of 5HT by platelets contributes to the pulmonary response to MCTP because fawn-hooded rats, which have a congenital defect in

which the ability of the platelet to take up and release 5HT is decreased (Raymond and Dodds, 1975), were no more susceptible to MCTP-induced toxicity than Sprague-Dawley rats (Hilliker et al., 1983a). Conversely, since platelets compete with the pulmonary vascular endothelium for uptake of 5HT (Steinberg and Das, 1980), and 5HT removal by endothelium is impaired following treatment with MCTP (Hilliker et al., 1983c), platelets could contain a greater amount of 5HT which would be available for release upon activation, thereby causing greater pulmonary vasoconstriction. This also does not appear to be true, as platelets from MCTP-treated rats contain no more 5HT than do platelets from control rats (Figure 17).

In addition to elevated pulmonary concentrations of 5HT causing vasoconstriction, it was hypothesized that greater accessibility of smooth muscle receptors or increased responsiveness of vascular smooth muscle to 5HT following MCTP treatment could contribute to pulmonary hypertension. Pulmonary vessel leak precedes pulmonary hypertension in MCT-treated (Sugita et al., 1983a) or MCTP-treated (Table 3) rats. This increased vascular permeability could increase the accessibility of smooth muscle receptors to 5HT originating from the blood. Conversely, it also seemed possible that 5HT could contribute to the observed vessel leak (DeClerck et al., 1984, 1985b). Pressor responses to 5HT were elevated in isolated, perfused lungs from MCTP-treated rats compared to control rats (Hilliker and Roth, 1985a), suggesting that MCTP treatment increases vascular responsiveness to 5HT. Therefore, 5HT could cause pulmonary vasoconstriction in MCTP-treated rats through greater interaction with 5HT receptors on vascular smooth muscle cells or through enhanced vasoconstriction due to hyperresponsiveness of the vasculature.

If 5HT were contributing to MCTP-induced pulmonary hypertension, co-treatment with an inhibitor of 5HT synthesis or with a 5HT receptor antagonist

may have reduced the resonse to MCTP. Co-treatment with para-chlorophenylalanine (PCPA), a 5HT synthesis inhibitor, decreased PAP and RVE in MCT-treated rats (Carrillo and Aviado, 1969; Tucker et al., 1982; Kay et al., 1985). It is possible that PCPA acted in these studies by decreasing 5HT-induced pulmonary vasoconstriction and/or lung leak. However, there are some potential problems with these three studies. One is that drug effectiveness (i.e., decreased synthesis of 5HT) was never confirmed. Secondly, animals treated with PCPA gained less weight than control animals, and it has been demonstrated that diminished body weight gain is associated with prevention of RVE and amelioration of lung injury in MCT- or MCTP-treated rats (Hayashi et al., 1979; Figure 7). It is also possible that in these studies PCPA might have exerted its protective effect by inhibiting the mixed function oxidase enzyme system, thereby inhibiting bioactivation of MCT. In support of this, PCPA was ineffective in reducing the severity of pleural effusion or in prolonging survival in MCTP-treated rats (Plestina and Stoner, 1972).

To examine whether 5HT was contributing to MCTP-induced pulmonary hypertension, MCTP-treated rats were co-treated with a 5HT receptor antagonist. 5HT-induced contraction of vascular smooth muscle is mediated by a population of 5HT receptors termed $5HT_2$ receptors (Cohen et al., 1981); therefore, the effect of ketanserin, which specifically antagonizes $5HT_2$ receptors (Leysen et al., 1980; Laduron et al., 1982), was determined in MCTP-treated rats. Ketanserin inhibited 5HT-induced contraction in isolated vessels (Van Nueten et al., 1981; Ogunbiyi and Eyre, 1984) without altering 5HT uptake into platelets (De Clerck et al., 1982a). Ketanserin has also been shown to reduce pulmonary arterial pressure in humans (Vincent et al., 1984). At the dosing regimen used in the present study, ketanserin inhibited the 5HT-induced shape change in platelet-rich plasma (Figure 19) and the increase in perfusion pressure induced by 5HT in

isolated lungs from MCTP-treated rats (Figure 20). However, ketanserin did not protect against the alterations in lung weight or vascular leakage of ^{125}I-albumin observed in MCTP-treated rats (Figure 21). RVE in MCTP-treated rats was also not attenuated by co-treatment with ketanserin.

Because many 5HT receptor antagonists have other pharmacological actions which do not involve 5HT receptors, in studies not presented here the effect of a structurally different 5HT receptor antagonist was also examined. Metergoline, which has been reported to bind to both $5HT_1$ and $5HT_2$ receptors (Leysen et al., 1980; Peroutka and Snyder, 1983), did not protect against MCTP-induced changes in lung weight or RVE (Ganey et al., 1986).

The results of these studies indicate that MCTP treatment does not alter platelet 5HT content, and that co-treatment with the $5HT_2$ receptor antagonist ketanserin does not attenuate the response to MCTP. This suggests that 5HT does not mediate lung injury or RVE in this model. Because RVE is presumed to be the result of sustained pulmonary hypertension in this model, these findings also suggest that 5HT is not responsible for the increased pulmonary vascular pressure due to MCTP.

B. Thromboxane A_2

A role for TxA_2 in MCTP-induced pulmonary hypertension was hypothesized based on a number of observations. Activation of platelets by interaction with injured vessels causes release of TxA_2 which can cause pulmonary vasoconstriction (Svensson et al., 1977; Salzman et al., 1980; McDonald et al., 1983; Farrukh et al., 1985). Thromboxane has been implicated in the pulmonary hypertension produced in isolated lungs by staphylococcal α-toxin (Seeger et al., 1984) and by platelets activated with platelet-activating factor (Heffner et al., 1983) or Staphylococcus aureus (Shoemaker et al., 1984). Additionally, both the stable metabolite of TxA_2 (TxB_2) and the endoperoxide precursors to TxA_2 (PGG_2

and PGH_2) are vasoactive. TxB_2 causes pulmonary vasoconstriction in isolated lung lobes (Kadowitz and Hyman, 1980) and in vivo (Friedman et al., 1979). PGH_2, which may also act at thromboxane receptors, contracts vascular smooth muscle (Hamberg et al., 1975a; Malmsten et al., 1976), and increases pulmonary vascular resistance (Kadowitz et al., 1977; Hyman et al., 1978). The endoperoxide analog and thromboxane mimic U46619 increased right ventricular pressure in this study, and the increase was greater in MCTP-treated rats than controls (Table 35). TxA_2 could also increase pulmonary vascular resistance by causing vessel leak independent of its properties of venoconstriction (Garcia-Szabo et al., 1983).

In addition to contributing to MCTP-induced pulmonary hypertension through increasing pulmonary vascular pressure or pulmonary vessel leak, it seemed possible that TxA_2 could promote platelet aggregation and release of other vasoactive mediators, permeability modifiers or growth factors. TxA_2 (or PGG_2 and PGH_2) is involved in the platelet aggregation response to some agents, although this involvement varies with the species from which platelets are obtained (Hamberg et al., 1974; Malmsten et al., 1975; Meyers et al., 1979; Leach and Thorburn, 1982; Parise et al., 1984). TxA_2 does not appear to be a necessary requirement for aggregation of rat platelets in response to arachidonic acid, collagen, or thrombin (Nishizawa et al., 1983; Emms and Lewis, 1986; Huzoor-Akbar and Anwer, 1986).

It also seemed possible that the vasocontrictive and pro-aggregatory properties of TxA_2 might be more effective in the face of decreased PGI_2 synthesis due to MCTP-induced endothelial cell injury. Injury to cultured endothelial cells by exposure to a variety of agents has been associated with altered synthesis and/or release of both PGI_2 and TxA_2. For example, injury induced by an immunologic stimulus (Goldsmith and McCormick, 1984) or by radiation (Rubin et al., 1985) stimulated release of 6-keto $PGF_{1\alpha}$ from endothelial

cells. Concentrations of hydrogen peroxide which were not cytotoxic either inhibited production of 6-keto $PGF_{1\alpha}$ from arachidonate in endothelial cells (Whorton et al., 1985) or caused a transient increase in 6-keto $PGF_{1\alpha}$ followed by diminished release in response to subsequent stimuli (Ager and Gordon, 1984). Endotoxin caused release of both 6-keto $PGF_{1\alpha}$ (Nawroth et al., 1984) and TxB_2 (Nawroth et al., 1985) from cultured endothelial cells. In isolated human umbilical veins, production of TxB_2 was stimulated to a greater extent than PGI_2 by balloon trauma (Mehta et al., 1982), and in vivo atherosclerosis is associated with increased PGI_2 biosynthesis (FitzGerald et al., 1984). So, if MCTP-induced endothelial cell injury either depressed production of PGI_2 or did not stimulate PGI_2 synthesis to an extent capable of opposing the action of increased concentrations of TxA_2, then TxA_2 could contribute to pulmonary hypertension.

In addition to having vasodilatory and anti-aggregatory properties which oppose the activities of TxA_2, it has been reported that PGI_2 decreased thromboxane production in response to endotoxin (Flynn and Demling, 1982) and that, in isolated lungs perfused with platelets, TxB_2 was only produced when PGI_2 synthesis was inhibited (Boyd and Eling, 1980). These results suggest that PGI_2 may in some way regulate TxA_2 synthesis as well as oppose the actions of TxA_2.

Thus, TxA_2 may contribute to MCTP-induced pulmonary hypertension by causing pulmonary vasoconstriction or pulmonary vascular leak, by promoting platelet activation and mediator release, or because of an imbalance in the relative activities of PGI_2 and TxA_2.

In proposing a possible role for TxA_2 in pulmonary hypertension due to MCTP, it was important to demonstrate that TxA_2 was present in the lungs of treated rats. MCT-induced pulmonary hypertension is associated with an elevation of TxB_2 and 6-keto $PGF_{1\alpha}$ in lavage fluid (Stenmark et al., 1985) and lung minces (Molteni et al., 1984, 1986). It was of interest to examine vascular

production of these prostanoids. Therefore, TxB_2 and 6-keto $PGF_{1\alpha}$ release from isolated lungs of MCTP-treated rats was measured at the onset of pulmonary hypertension (day 7) and once pulmonary hypertension was well established (day 14).

In isolated lungs perfused with a cell-free buffer, the release of 6-keto $PGF_{1\alpha}$ was not different in lungs from rats treated 7 days earlier with MCTP and control rats (Figure 22A). The release of TxB_2 was also similar in lungs from treated and control rats at this time (Figure 22B). Pulmonary injury was evidenced in treated rats at this time by an increase in lavage LDH activity and in lung weight (Table 19), and inflow perfusion pressure was also elevated (Table 20), reflecting the pulmonary hypertensive condition of treated rats. However, these changes were not associated with altered production or release of 6-keto $PGF_{1\alpha}$ or TxB_2.

Fourteen days following treatment with MCTP, lung injury was still apparent and RVE was observed (Table 21) suggesting that pulmonary hypertension was established. In support of this, inflow perfusion pressure was higher in lungs of MCTP-treated rats (Table 22). At this time, 6-keto $PGF_{1\alpha}$ release from isolated, buffer-perfused lungs of MCTP-treated rats tended to be higher than from lungs of controls, although this difference did not reach statistical significance (Figure 24A). When presented with arachidonic acid, lungs from both control and treated rats released more 6-keto $PGF_{1\alpha}$, suggesting that MCTP treatment did not impair the lung's ability to synthesize PGI_2.

TxB_2 release was greater from lungs of rats treated with MCTP 14 days earlier than from control rats, both prior to and during arachidonate infusion (Figure 24B). What was the source of TxB_2 in these buffer-perfused lungs? TxB_2 could have derived from platelets adhered to vessels, and greater platelet adherence due to endothelial cell damage may explain the elevated TxB_2 release

in lungs of MCTP-treated rats relative to controls. Alternatively, TxB_2 could have derived from endothelial cells (Ingerman-Wojenski, 1981; Goldsmith and Needleman, 1982) or fibroblasts (Ali et al., 1980; Ody et al., 1982; Menconi et al., 1984), both of which may be proliferating in lungs of treated rats at this time. Results from lungs deliberately made edematous by increasing outflow pressure (Figure 25) suggest that a propensity to accumulate fluid, such as is seen in lungs from MCTP-treated rats (Table 21), may contribute to elevated release of TxB_2 from isolated lungs. In response to arachidonate stimulus, release of TxB_2 increased in lungs from both control and treated rats, suggesting that MCTP treatment did not impair the lung's capacity to synthesize TxA_2.

These results suggested that MCTP-induced pulmonary hypertension was associated with elevated production in the lung of TxB_2 and not 6-keto $PGF_{1\alpha}$. This supported the possibility that the balance of TxA_2 and PGI_2 favored the vasoconstrictory prostanoid TxA_2 in lungs of MCTP-treated rats. Since the platelet is the major source of TxA_2 in vivo (Needleman et al., 1976), and since platelet/vessel wall interaction can promote TxA_2 release, it was of interest to examine release of TxB_2 and 6-keto $PGF_{1\alpha}$ in lungs perfused with blood.

At day 7, lung weight was elevated in MCTP-treated rats, but RVE was not apparent (Table 23). Inflow perfusion pressure tended to be higher in lungs from treated rats, but this difference was not significant (Table 24). Similar to results in buffer-perfused lungs, at day 7 there were no differences in the effluent concentrations of either TxB_2 or 6-keto $PGF_{1\alpha}$ between lungs from control and treated rats (Figure 26). The change in platelet number between the inflow and effluent perfusates was not different from zero for either group (Table 24), suggesting that significant numbers of platelets were not adhering to the vasculature during perfusion.

At day 14, lung weight was elevated and RVE was observed (Table 25). Inflow perfusion pressure was higher in lungs from MCTP-treated rats, reflecting the pulmonary hypertensive condition of these rats (Table 26). At this time, the effluent concentration of 6-keto $PGF_{1\alpha}$ was not different in lungs from MCTP-treated rats and control rats (Figure 27A). However, the concentration of TxB_2 in the effluent of lungs from MCTP-treated rats was significantly greater than controls by 3 minutes of blood perfusion (Figure 27B). TxB_2 in the effluent of lungs from rats treated 14 days earlier with MCTP increased during the perfusion. This increase was not observed in controls. The reason for this 3-minute delay until the observed increase in TxB_2 is unclear. One possible explanation is that a period of time may be required for activation of platelets encountering injured endothelium. Platelets in the blood perfusate were probably not adhering to the vasculature, as the platelet number was not different in the inflow and the effluent perfusate (Table 26), however activation of platelets in the absence of aggregation and adherence may have occurred.

Besides platelets, other sources of TxB_2, such as endothelial cells (Goldsmith and Needleman, 1982), leukocytes (Goldstein et al., 1978), or fibroblasts (Menconi et al., 1984) may also have contributed to the plasma effluent TxB_2 concentration. There are two suggestions that the blood was a partial source of TxB_2. The first is that the release of TxB_2 was not different in lungs from treated and control rats during the buffer pre-perfusion (data not shown). The second is drawn from a comparison of the magnitude of increase in TxB_2 release in blood- and buffer-perfused lungs (Figures 27 and 24). The TxB_2 concentration in the effluent of lungs from rats treated 14 days earlier with MCTP was 0.2 ng/ml greater than that of controls when lungs were perfused with buffer (Figure 24B). When perfused with blood, the concentration of TxB_2 in the plasma effluent of lungs from MCTP-treated rats was 1.6 ng/ml greater than

control (Figure 27B). Thus, substantially more TxB_2 was released when lungs were perfused with blood. This argues against lung tissue as the sole source of increased release of thromboxane caused by MCTP treatment. It also seems unlikely that the elevated TxB_2 concentration in lungs from MCTP-treated rats could be explained by less efficient removal of TxB_2 by these lungs because the initial extraction of TxB_2 was not different from controls (Figure 28).

Thus, MCTP-induced pulmonary hypertension was associated with an increased release of TxB_2 and unaltered release of 6-keto $PGF_{1\alpha}$ from isolated lungs perfused with either a cell-free buffer or blood. The magnitude of the increased release of TxB_2 relative to controls was greater in lungs perfused with blood, suggesting a source of the TxB_2 was within the blood. This possibly could be the platelets. These findings indicate that, in isolated lungs of MCTP-treated rats which have developed RVE (and presumably elevated pulmonary arterial pressures), the balance of TxA_2 and PGI_2 is tipped in favor of TxA_2. These results suggest that if events occurring in the isolated lung reflect events occurring in vivo, TxA_2 could be contributing to MCTP-induced pulmonary hypertension due to vasoconstriction from elevated pulmonary concentrations of TxA_2.

In addition to increased release of TxA_2 in isolated lungs, it was of interest to examine whether MCTP treatment in vivo altered platelets so that they released more TxA_2 upon activation. Some human diseases that involve vascular complications, such as diabetes (DiMinno et al., 1985) and Kawasaki disease (Hidaka et al., 1983) are associated with increased platelet production of TxA_2. Platelets from spontaneously hypertensive rats also synthesize more TxA_2 than platelets from normotensive rats (DeClerk et al., 1982b; Huzoor-Akbar and Anwer, 1986). Therefore, it seemed possible that MCTP might alter platelet production of TxB_2.

The concentration of TxB_2 was higher in unstimulated, platelet-rich plasma (PRP) from MCTP-treated rats relative to controls at day 1, but not at any other time after treatment (Figure 29A). Although lung injury was not assessed in the present study at this time, in a different study, vascular leak was not elevated one day after treatment with MCTP (Bruner et al., 1986). In addition, injury reported here at day 3 (Table 3) or 4 (Table 27) was relatively mild so that major injury would not be expected at day 1. Thus, the basal concentration of TxB_2 in PRP was likely elevated before the onset of major injury. This could be caused by an MCTP-induced alteration in the platelets such that they released TxA_2 more readily during the preparation of PRP. Alternatively, the observed increase in TxB_2 in unstimulated PRP could be derived from other cellular sources of TxB_2 in the blood.

The release of TxB_2 in response to arachidonic acid-induced platelet aggregation was not higher in PRP from MCTP-treated rats at any time after treatment (Figures 29B and 29C). Thus, administration of MCTP in vivo does not increase platelet production of TxA_2. Relatively high concentrations (1 mg/ml) of MCTP added directly to PRP in vitro abolished the aggregation response and decreased TxB_2 production, but lower concentrations of MCTP did not affect either the aggregation response or the release of TxB_2 (Table 29). This indicates that moderate concentrations of MCTP do not directly affect platelets.

In contrast to previous findings (Hilliker et al., 1983b), the aggregation response to arachidonic acid was not different in PRP from treated and control rats (Table 28). Hilliker and coworkers (1983b) demonstrated a 36% decrease in maximal aggregation and a 40% decrease in the rate of aggregation in response to arachidonic acid in PRP from rats treated 14 days earlier with MCTP. The reason for the different results obtained here is not obvious.

Thus, although MCTP treatment resulted in elevated TxB_2 release by isolated lungs, it did not enhance TxB_2 production by platelets and did not alter platelet aggregation responses.

If TxA_2 is involved in MCTP-induced pulmonary hypertension, co-treatment with a drug which inhibits the biosynthesis of TxA_2 or interferes with its activity may have reduced the pulmonary hypertensive response to MCTP. Some drugs which inhibit prostaglandin biosynthesis, but also have other actions unrelated to prostaglandins, have afforded partial protection against the toxicity of MCTP. Hydralazine, which inhibits platelet thromboxane synthesis (Greenwald et al., 1978), attenuated the development of RVE and the increase in lavage protein concentration in MCTP-treated rats (Hilliker and Roth, 1984). Sulphinpyrazone, which also inhibits platelet prostaglandin biosynthesis (Livio et al., 1980), prevented the development of RVE, but did not alter the indices of lung injury (Hilliker and Roth, 1984). However, inhibition of prostaglandin biosynthesis was not confirmed in these studies, and protective effects may have been due to some other action (for example, vasodilation) of these drugs. In addition, sulphinpyrazone depressed body weight gain, and this effect may have contributed to the observed protection. To examine the involvement of TxA_2 in MCTP-induced pulmonary hypertension more closely, rats were co-treated with either ibuprofen, Dazmegrel, or L-640,036.

Ibuprofen inhibits the enzyme cyclooxygenase (Longenecker et al., 1985) which converts arachidonic acid to the prostaglandin endoperoxide which is then converted to TxA_2, PGL_2, and prostaglandins of the A-F series (Figure 2). Ibuprofen has also been reported to inhibit platelet function (McIntyre and Philp, 1977). Treatment with ibuprofen significantly decreased pulmonary hypertension in a porcine model of acute respiratory failure (Kopolovic et al., 1984). However, co-treatment with doses of ibuprofen which decreased platelet function (Table 30)

and circulating plasma thromboxane concentration did not alter MCTP-induced RVE or increased lung weight (Table 31). This is consistent with the finding that co-treatment with indomethacin, another cyclooxygenase inhibitor, did not alter MCT-induced toxicity (Stenmark et al., 1985).

Inhibition of cyclooxygenase also suppresses synthesis of PGI_2 (Figure 2), the vasodilatory and anti-aggregatory action of which may be beneficial in relieving pulmonary hypertension. Treatment with PGI_2 has afforded some relief to patients suffering from primary pulmonary hypertension (Higenbottom et al., 1984). In addition, PGI_2 infused into the pulmonary artery acutely reduced pulmonary arterial pressure in monocrotaline-treated rats (Bowdy et al., 1986) and dogs (Czer et al., 1986). PGE_1, another product of the cyclooxygenase enzyme, has also been shown to reverse the pulmonary hypertension in rats fed C. spectabilis seeds (Roum et al., 1983). Inhibition of the effects of PGI_2 or PGE_1 by treatment with ibuprofen could have masked any protective effect of inhibition of thromboxane synthesis, therefore, the effect of a specific thromboxane synthetase inhibitor was determined in MCTP-treated rats. Treatment with Dazmegrel (UK38485) decreased serum TxB_2 levels in animals (Parry et al., 1982) and man (Fischer et al., 1983), but had no effect on the plasma concentration of 6-keto-$PGF_{1\alpha}$ (Fischer et al., 1983). Similar results were seen here (Table 32). Despite a depressed plasma concentration of TxB_2, co-treatment with Dazmegrel did not alter MCTP-induced lung injury at day 7 (Table 33) or 14 (Table 34), or MCTP-induced RVE at day 14 (Figure 31). This is consistent with the recent report that Dazmegrel did not alter toxicity caused by administering the parent compound, monocrotaline (Langleben et al., 1986).

The endoperoxide precursor to TxA_2, PGH_2, has been reported to have pro-aggregatory (Hamberg et al., 1974) and pulmonary vasoconstrictive actions (Kadowitz et al., 1977) similar to TxA_2 itself, and is thought to act at the same

receptor as TxA_2. Therefore, treatment with a thromboxane synthetase inhibitor may not completely prevent this activity. Accordingly, the effect of co-treatment with a thromboxane receptor antagonist on MCTP-induced toxicity was determined. L-640,035 inhibits platelet aggregation in guinea pigs and rabbits in vivo, and human platelet aggregation in vitro in response to the prostaglandin endoperoxide analogue U44069, to arachidonate, or to collagen, but not to ADP (Chan et al., 1986). The U44069-induced increase in pulmonary resistance in guinea pigs and dogs in vivo was also inhibited by L-640,035 (Carrier et al., 1984). Treatment with L-640,035 inhibited the increase in right ventricular pressure induced by U46619 in anesthetized rats (Figure 32, Table 35). Co-treatment with L-640,035 did not attenuate the MCTP-induced elevation in lung weight or LDH activity and protein concentration in lavage fluid (Table 36), nor did it affect the MCTP-induced elevation in pulmonary arterial pressure at day 14 (Figure 33).

An interesting finding was the greater response to U46619 in MCTP/VEH-treated rats relative to DMF/VEH controls (Table 35). This is consistent with the finding of increased vascular responsiveness in isolated, perfused lungs from MCT- (Gillespie et al., 1986) or MCTP-treated rats (Hilliker and Roth, 1985a). Co-treatment with L-640,035 essentially returned the response to U46619 in MCTP-treated rats to that seen in DMF control rats. This raises the possibility that, if there were an elevated concentration of TxA_2 in the pulmonary vasculature of MCTP-treated rats, then the greater vascular responsiveness might allow thromboxane to contribute to pulmonary hypertension despite the degree of receptor antagonism achieved in this study. It is unclear whether the pulmonary concentration of TxA_2 is elevated in MCTP-treated rats. Results in isolated, perfused lungs would suggest that pulmonary concentrations of TxB_2 may be elevated 14 days after treatment with MCTP, however, plasma concentrations of

TxB_2 were no greater in MCTP-treated rats at day 14 than in DMF controls (Table 32).

In summary, administration of MCTP to rats was associated with elevated release of TxB_2 from isolated, perfused lungs at day 14 when pulmonary hypertension was established, but not at day 7 during the onset of pulmonary hypertension. The MCTP-induced increase in TxB_2 was greater in lungs perfused with blood than in lungs perfused with buffer, suggesting that part of the TxB_2 was derived from the blood. The release of 6-keto $PGF_{1\alpha}$ was not affected by treatment with MCTP. Platelets from MCTP-treated rats did not produce more TxB_2 during aggregation with arachidonic acid than platelets from control rats, nor were they altered in responsiveness to arachidonic acid. Co-treatment with drugs which inhibit the biosynthesis of TxA_2 (ibuprofen or Dazmegrel) or with a TxA_2 receptor antagonist (L-640,035) did not attenuate the lung injury or pulmonary hypertension caused by MCTP. These results suggest that TxA_2 is not necessary for the pulmonary hypertensive response to MCTP.

SUMMARY AND CONCLUSIONS

These studies were undertaken to examine the role of the platelet and platelet mediators in the pulmonary hypertension caused by MCTP. When rats treated with MCTP on day 0 were moderately depleted of platelets from days 6-8, pulmonary hypertension and RVE did not develop by day 14. MCTP-induced lung injury at this time was not affected by platelet depletion. These results suggested that the platelet was contributing in some way to the pulmonary hypertensive response to MCTP. One mechanism by which the platelet may have been involved was through release of vasoactive mediators. Two such mediators, 5HT and TxA_2, were examined as possibly playing a role in the response to MCTP.

Although 5HT causes pulmonary vasoconstriction in rats and increases vascular permeability, co-treatment with a 5HT receptor antagonist did not attenuate the lung injury, vessel leak, or RVE caused by MCTP. Treatment with MCTP also did not alter platelet content of 5HT. These results suggest that 5HT is not a necessary contributor to MCTP-induced toxicity.

Release of TxB_2, a stable metabolite of TxA_2, was elevated in lungs of MCTP-treated rats relative to controls. In addition, the Tx mimic U46619 produced pulmonary vasoconstriction in anesthetized rats, and this response was enhanced in rats treated 14 days earlier with MCTP. These results suggested that the concentration of TxA_2 may be elevated in lungs of MCTP-treated rats, and that these lungs may respond to TxA_2 with greater vasoconstriction than controls. Treatment with MCTP did not alter arachidonic acid-induced aggregation or

release of TxB_2 from platelets, suggesting that in treated rats platelet activation would not be accompanied by a greater release of TxA_2.

Co-treatment with either a cyclooxygenase inhibitor (ibuprofen), a thromboxane synthetase inhibitor (Dazmegrel) or a thromboxane receptor antagonist (L-640,035) did not alter the lung injury, pulmonary hypertension, or RVE caused by MCTP. These results suggested that TxA_2 was also not necessary for the development of pulmonary hypertension due to treatment with MCTP.

By what mechanism does the platelet contribute to MCTP-induced pulmonary hypertension? At this time, the answer to that question is unknown. One potentially interesting area of investigation was not addressed in these studies: the possible contribution of platelet-derived growth factors, including PDGF. In light of the observation of pronounced cell proliferation in lungs of MCTP-treated rats, and studies suggesting that inhibition of polyamine biosynthesis attenuates the pneumotoxic and pulmonary hypertensive response to MCTP, investigation into the role of growth factors may provide some important information into the mechanism by which the platelet contributes to MCTP toxicity.

BIBLIOGRAPHY

BIBLIOGRAPHY

Adams, R. and Rodgers, E.F.: The structure of monocrotaline, the alkaloid in Crotalaria spectabilis and Crotalaria retusa. I. J. Am. Chem. Soc. 61: 2815-2819, 1939.

Ager, A. and Gordon, J.L.: Differential effects of hydrogen peroxide on indices of endothelial cell function. J. Exp. Med. 159: 592-603, 1984.

Ahmed, T. and Sackner, M.A.: Increased serum copper in primary pulmonary hypertension: A possible pathogenic link. Respiration 47: 243-246, 1985.

Allen, J.R., Carstens, L.A. and Knezevic, A.L.: Crotalaria spectabilis intoxication in Rhesus monkeys. Am. J. Vet. Res. 26: 753-757, 1965.

Allen, J.R., Chesney, C.F. and Frazee, W.J.: Modifications of pyrrolizinde alkaloid intoxication resulting from altered hepatic microsomal enzymes. Toxicol. Appl. Pharmacol. 23: 470-479, 1972.

Allen, J.R., Childs, G.R. and Cravens, W.W.: Crotalaria spectabilis toxicity in chickens. Proc. Soc. Exp. Biol. Med. 104: 434-436, 1960.

Allen, J.R., Lalich, J.J. and Schmittle, S.C.: Crotalaria spectabilis induced cirrhosis in turkeys. Lab. Invest. 12: 512-517, 1963.

Ali, A.E., Barrett, J.C. and Eling, T.E.: Prostaglandin and thromboxane production by fibroblasts and vascular endothelial cells. Adv. in Prostaglandin and Thromboxane Res. 6: 533-535, 1980.

Altiere, R.J., Hennig, B., Gillespie, M.N. and Olson, J.W.: Enhanced ornithine decarboxylase (ODC) activity in pulmonary artery endothelial cells following exposure to monocrotaline (MCT) metabolites. Fed. Proc. 45: 555, 1986a.

Altiere, R.J., Olson, J.W. and Gillespie, M.N.: Altered pulmonary vascular smooth muscle responsiveness in monocrotaline-induced pulmonary hypertension. J. Pharmacol. Exp. Ther. 236: 390-395, 1986b.

Becker, R.B., Neal, W.M., Arnold, P.T.D. and Shealy, A.L.: A study of the palatability and possible toxicity of 11 species of Crotalaria especially of C. spectabilis. J. Agric. Res. 50: 911-922, 1935.

Benveniste, J. and Chignard, M.: A role for PAF-acether (platelet-activating factor) in platelet-dependent vascular diseases. Circ. 72: 713-717, 1985.

Bergemeyer, H.U. and Bernt, E.: Lactate dehydrogenase UV assay with pyruvate and NADPH. In: Methods of Enzymatic Analysis, Vol. 2 (H.U. Bergmeyer, ed.). New York: Academic press, 1974, p. 574-579.

Bernard, G.R. and Brigham, K.L.: The adult respiratory distress syndrome. Ann. Rev. Med. 36: 195-205, 1985.

Bø, G. and Hognestad, J.: Effects on the pulmonary circulation of suddenly induced intravascular aggregation of blood platelets. Acta Physiol. Scand. 85: 523-531, 1972.

Born, G.V.R.: Observations on the change in shape of blood platelets brought about by adenosine diphosphate. J. Physiol. 209: 487-511, 1970.

Born, G.V.R. and Cross, M.J.: The aggregation of blood platelets. J. Physiol. 168: 178-195, 1963.

Born, G.V.R., Juengijaroen, K. and Michal, F.: Relative activities on and uptake by human blood platelets of 5-hydroxytryptamine and several analogues. Br. J. Pharmacol. 44: 117-139, 1972.

Bourgain, R.H., Andries, R., Finne, E., Driessche, R.J.V. and Bernard, P.J.: The effect of the inhibition of PGI_2 synthetase and lipoxygenase and of arachidonic acid on platelet-vessel wall interaction. Arch. int. Pharmacodyn. Ther. 259: 305-307, 1982.

Bowdy, B.D., Dyer, K.K., Olson, J.W., Altiere, R.J. and Gillespie, M.N.: Hemodynamic effects of PGI_2 in rats with monocrotaline (MCT)-induced pulmonary hypertension. Fed. Proc. 45: 555, 1986.

Boyd, J.A. and Eling, T.E.: Prostaglandin release and the interaction of platelets with the pulmonary vasculature of rat and guinea pig. Thromb. Res. 19: 239-248, 1980.

Bras, G., Jelliffe, D.B. and Stuart, K.L.: Veno-occlusive disease of the liver with non-portal type cirrhosis occurring in Jamaica. Arch. Pathol. 57: 285-300, 1954.

Brecher, G. and Cronkite, E.P.: Morphology and enumeration of human blood platelets. J. Appl. Physiol. 3: 365-377, 1950.

Breuer, J., Meschig, R., Breuer, H-W.M. and Arnold, G.: Effects of serotonin on the cardiopulmonary circulatory system with and without 5-HT_2-receptor blockade by ketanserin. J. Cardiovasc. Pharmacol. 7(Suppl. 7): 564-566, 1985.

Bruner, L.H.: The role of immune effectors in monocrotaline pyrrole-induced pulmonary injury. A dissertation submitted to Michigan State University, 1986.

Bruner, L.H., Carpenter, L.J., Hamlow, P. and Roth, R.A.: Effect of a mixed function oxidase inducer and inhibitor on monocrotaline pyrrole pneumotoxicity. Toxicol. Appl. Pharmacol., in press, 1986.

Bruner, L.H., Hilliker, K.S. and Roth, R.A.: Pulmonary hypertension and ECG changes from monocrotaline pyrrole in the rat. Am. J. Physiol. 245 (Heart Circ. Physiol. 14): H300-H306, 1983.

Bull, L.B., Culvenor, C.C.J. and Dick, A.T.: Historical and geographic survey of pyrrozlidine alkaloidosis. In: The Pyrrolizidine Alkaloids A. Neuberger and E.L. Tatum, eds.). Amsterdam: North-Holland, 1968.

Butler, W.H.: An ultrastructural study of the pulmonary lesion induced by pyrrole derivatives of the pyrrolizidine alkaloids. J. Pathol. 102: 15-19, 1970.

Butler, W.H., Mattocks, A.R. and Barnes, J.M.: Lesions in the liver and lungs of rats given pyrrole derivatives of pyrrolizidine alkaloids. J. Pathol. 100: 169-175, 1970.

Camussi, G., Bussolino, F., Tetta, C., Brusca, R. and Ragni, R.: The binding of platelet-activating factor (PAF) to polymorphonuclear neutrophils (PMN) as a trigger to the immune-induced PMN aggregation. Panminerva Medica 22: 1-5, 1980.

Carillo, L. and Aviado, D.M.: Monocrotaline-induced pulmonary hypertension and p-chlorophenylalanine (PCPA). Lab. Invest. 20: 243-248, 1969.

Carrier, R., Cragoe, E.J., Ethier, D., Ford-Hutchinson, A.W., Girard, Y., Hall, R.A., Hamel, P., Rokach, J., Share, N.N., Stone, C.A. and Yusko, P.: Studies on L-640,035: A novel antagonist of contractile prostanoids in the lung. Br. J. Pharmacol. 82: 389-395, 1984.

Carstens, L.A. and Allen, J.R.: Arterial degeneration and glomerular hyalinization in the kidney of monocrotaline-intoxicated rats. Am. J. Pathol. 60: 75-84, 1970.

Chan, C.C., Nathaniel, D.J., Yusko, P.J., Hall, R.A. and Ford-Hutchinson, A.W.: Inhibition of prostanoid-mediated platelet aggregation in vivo and in vitro by 3-hydroxymethyl-dibenzo(b,f)thiepin 5,5-dioxide (L-640,035). J. Pharmacol. Exp. Ther. 229: 276-282, 1984.

Cheeke, P.R. and Pierson-Goeger, M.L.: Toxicity of Senecio jacobaea and pyrrolizidine alkaloids in various laboratory animals and avian species. Toxicol. Lett. 18: 343-349, 1983.

Chesney, C.F. and Allen, J.R.: Endocardial fibrosis associated with monocrotaline-induced pulmonary hypertension in nonhuman primates (Macaca arctoides). Am. J. Vet. Res. 34: 1577-1581, 1973a.

Chesney, C.F. and Allen, J.R.: Resistance of the guinea pig to pyrrolizidine alkaloid intoxication. Toxicol. Appl. Pharmacol. 26: 385-392, 1973b.

Chesney, C.F., Allen, J.R. and Hsu, L.C.: Right ventricular hypertrophy in monocrotaline pyrrole-treated rats. Exp. Mol. Pharmacol. 20: 257-268, 1974a.

Chesney, C.F., Hsu, I.C. and Allen, J.R.: Modifications of the in vitro metabolism of the hepatotoxic pyrrolizidine alkaloid, monocrotaline. Res. Commun. Chem. Pathol. Pharmacol. 8: 567-570, 1974b.

Chignard, M., LeCouedic, J.P., Tence, M., Vargaftig, B.B. and Benveniste, J.: The role of platelet-activating factor in platelet aggregation. Nature 279: 799-800, 1979.

Coflesky, J.T., Szarek, J.L., Langleben, D., Jones, R.C., Reid, L. and Evans, J.N.: Mechanical properties and structure of pulmonary arteries (PA) in monocrotaline (MCT)-induced pulmonary hypertension. Fed. Proc. 44: 1753, 1985.

Cohen, M.L., Fuller, R.W. and Wiley, K.S.: Evidence for 5-HT_2 receptors mediating contraction in vascular smooth muscle. J. Pharmacol. Exp. Ther. 218: 421-425, 1981.

Cook, J.W., Duffy, E. and Schoenthal, R.: Primary liver tumors in rats following feeding with alkaloids of Senecio jacobaea. Brit. J. Cancer 4: 405-410, 1950.

Costa, J.L., Kirk, K.L. and Stark, H.: Uptake of 6-fluoro-5-hydroxytryptamine and 4,6-difluoro-5-hydroxytryptamine into releasable and non-releasable compartments of human platelets. Br. J. Pharmacol. 75: 237-242, 1982.

Creese, B.R., Bach, M.K., Fitzpatrick, F.A. and Bothwell, W.M.: Leukotriene-induced contraction and thromboxane production in guinea-pig lung parenchymal strips. Eur. J. Pharmacol. 102: 197-204, 1984.

Culvenor, C.C.J. and Dal Bon, R.: Carbonyl stretching frequencies of pyrrolizidine alkaloids. Austr. J. Chem. 17: 1296-1300, 1964.

Culvenor, C.C.J., Edgar, J.A., Jago, M.V., Outteridge, A., Peterson, J.E. and Smith, L.W.: Hepato- and pneumotoxicity of pyrrolizidine alkaloids and derivatives in relation to molecular structure. Chem.-Biol. Interactions 12: 299-324, 1976.

Culvenor, C.C.J., Edgar, J.A., Smith, L.W. and Tweedale, H.J.: Dihydropyrrolizidines. III. Preparation and reactions of derivatives related to pyrrolizidine alkaloids. Austr. J. Chem. 23: 1853-1867, 1970.

Czer, G.T., Marsh, J., Konopka, R. and Moser, K.M.: Low-dose PGI_2 prevents monocrotaline-induced thromboxane production and lung injury. J. Appl. Physiol. 60: 464-471, 1986.

Dahm, L.J., Bruner, L.H. and Roth, R.A.: Superoxide (O_2^-) production by bronchoalveolar cells harvested from monocrotaline pyrrole (MCTP)-treated rats. Fed. Proc. 45: 220, 1986.

Dale, C. and Hurley, J.V.: An electron-microscope study of the mechanism of bleeding in experimental thrombocytopenia. J. Pathol. 121: 193-204, 1977.

DeClerck, F., David, J.-L. and Janssen, P.A.J.: Inhibition of 5-hydroxytryptamine-induced and -amplified human platelet aggregation by ketanserin (R 41 468), a selective 5-HT_2 receptor antagonist. Agents Actions 12: 388-397, 1982a.

DeClerck, F., Loots, N., Somers, Y., Van Gorp, L., Verheyen, A. and Wouters, L.: Thromboxane A_2-induced vascular endothelial cell damage and respiratory smooth muscle cell contraction: Inhibition by flunarizine, a Ca^{++}-overload blocker. Arch. int. Pharmacodyn. 274: 4-23, 1985a.

DeClerck, F., Van Gorp, L., Beetens, J. and Reneman, T.R.S.: Platelet-mediated vascular permeability in the rat: A predominant role for 5-hydroxytryptamine. Thromb. Res. 38: 321-339, 1985b.

DeClerck, F., Van Gorp, L., Xhonneux, B., Somers, Y. and Wouters, L.: Enhanced platelet turnover and prostaglandin production in spontaneously hypertensive rats. Thromb. Res. 27: 243-249, 1982b.

DeClerck, F. and Van Nueten, J.M.: Platelet-mediated vascular contractions: Inhibition of the serotonergic component by ketanserin. Thromb. Res. 27: 713-727, 1982.

DeClerck, F., Van Nueten, J.M. and Reneman, R.S.: Platelet-vessel wall interaction: Implication of 5-hydroxytryptamine. Agents Actions 15: 612-626, 1984.

Deuel, T.F. and Huang, J.S.: Platelet-derived growth factor. J. Clin. Invest. 74: 669-676, 1984.

Dickinson, J.O.: Release of pyrrolizidine alkaloids into milk. Proc. West. Pharmacol. Soc. 23: 377-379, 1980.

Dickinson, J.O., Cooke, M.P., King, R.R. and Mohamed, P.A.: Milk transfer of pyrrolizidine alkaloids in cattle. J. Am. Vet. Med. Assoc. 169: 1192-1196, 1976.

Dienzer, M.L., Thompson, D.M., Burgett, D.M. and Isaacson, D.L.: Pyrrolizidine alkaloids: Their occurrence in honey from tansy ragwort (Senecio jacobaea L.). Science 195: 497-499, 1977.

DiMinno, G., Silver, M.J., Cerbone, A.M., Riccardi, G., Rivellese, A., Mancini, M. and Thiagarajan, P.: Increased binding of fibrinogen to platelets in diabetes: The role of prostaglandins and thromboxane. Blood 65: 156-162, 1985.

Drummond, A.H. and Gordon, J.L.: Specific binding sites for 5-hydroxytryptamine on rat blood platelets. Biochem. J. 150: 129-132, 1975.

Emmel, M.W., Sanders, D.A. and Healey, W.W.: Crotalaria spectabilis Roth seed poisoning in swine. J. Am. Vet. Med. Assoc. 86: 43-54, 1935.

Emms, H. and Lewis, G.P.: The roles of prostaglandin endoperoxides, thromboxane A_2 and adenosine diphosphate in collagen-induced aggregation in man and the rat. Br. J. Pharmacol. 87: 109-115, 1986.

Farrukh, I.S., Michael, J.R., Summer, W.R., Adkinson, N.F. Jr. and Gurtner, G.H.: Thromboxane-induced pulmonary vasoconstriction: Involvement of calcium. J. Appl. Physiol. 58: 34-44, 1985.

Feddersen, C.O., Mathias, M., Murphy, R.C., Reeves, J.T. and Voelkel, N.F.: Leukotriene E_4 causes pulmonary vasoconstriction, not inhibited by meclofenamate. Prostaglandins 26: 869-883, 1983.

Fernandez-Bonetti, P., Lupi-Heriera, E., Martinez-Guerra, M.L., Barrios, R., Sloane, M. and Sandoval, J.: Peripheral airways obstruction in idiopathic pulmonary artery hypertension (primary). Chest 83: 732-738, 1983.

Findlay, S.R., Lichtenstein, L.M., Hanahan, D.J. and Pinckard, R.N.: Contraction of guinea pig ileal smooth muscle by acetyl glyceryl ether phosphorylcholine. Am. J. Physiol. 241: C130-C133, 1981.

Fine, K.M., Dupont, J. and Mathias, M.M.: Rat platelet prostaglandin, cyclic AMP and lipid response to variations in dietary fat. J. Nutrition 111: 699-707, 1981.

Fischer, S., Struppler, M., Bohlig, B., Bernutz, C., Wober, W. and Weber, P.C.: The influence of selective thromboxane synthetase inhibition with a novel imidazole derivative, UK-38,485, on prostanoid formation in man. Circulation 68: 821-826, 1983.

FitzGerald, G.A., Smith, B., Pedersen, A.K. and Brash, A.R.: Increased prostacyclin biosynthesis in patients with severe atherosclerosis and platelet activation. N. Eng. J. Med. 310: 1065-1068, 1984.

Flynn, J.T. and Demling, R.H.: Inhibition of endogenous thromboxane synthesis by exogenous prostacyclin during endotoxemia in conscious sheep. Adv. in Shock Res. 7: 199-207, 1982.

Fox, D.W., Hart, M.C., Bergeson, P.S., Jarrett, P.B., Stillman, A.E. and Huxtable, R.J.: Pyrrolizidine (Senecio) intoxication mimicking Reye's syndrome. J. Pediatrics 93: 980-982, 1978.

Friedman, L.S., Fitzpatrick, T.M., Bloom, M.F., Ramwell, P.W., Rose, J.C. and Kot, P.A.: Cardiovascular and pulmonary effects of thromboxane B_2 in the dog. Circ. Res. 44: 748-751, 1979.

Fulton, R.M., Hutchinson, E.C. and Jones, A.M.: Ventricular weight in cardiac hypertrophy. Br. Heart J. 14: 413-420, 1952.

Ganey, P.E., Fink, G.D. and Roth, R.A.: The effect of dietary restriction and altered sodium intake on the cardiopulmonary toxicity of monocrotaline pyrrole. Toxicol. Appl. Pharmacol. 78: 55-62, 1985.

Ganey, P.E., Sprugel, K.H., Boner, K.E. and Roth, R.A.: Monocrotaline pyrrole-induced cardiopulmonary toxicity is not altered by metergoline or ketanserin. J. Pharmacol. Exp. Ther. 237: 226-231, 1986.

Garcia-Szabo, R.R., Peterson, M.B., Watkins, W.D., Bizios, R., Kong, D.L. and Malik, A.B.: Thromboxane generation after thrombin. Circ. Res. 53: 214-222, 1983.

Gardiner, M.R., Royce, R. and Bobor, A.: Studies on Crotalaria crispata, a newly recognized cause of Kimberley horse disease. J. Pathol. Bact. 89: 43-55, 1965.

Ghodzi, R. and Will, J.A.: Changes in pulmonary structure and function induced by monocrotaline intoxication. Am. J. Physiol. 240 (Heart Circ. Physiol. 9): H149-H155, 1981.

Gillespie, M.N., Fredrick, W.B., Altiere, R.J., Olson, J.W. and Kimmel, E.C.: Pulmonary mechanical, ventilatory and gas exchange abnormalities in rats with monocrotaline-induced pulmonary hypertension. Exp. Lung Res. 8: 189-197, 1985.

Gillespie, M.N., Olson, J.W., Reinsel, C.N., O'Connor, W.N. and Altiere, R.J.: Vascular hyperresponsiveness in perfused lungs from monocrotaline-treated rats. Am. J. Physiol., in press, 1986.

Gillis, C.N., Huxtable, R.J. and Roth, R.A.: Effects of monocrotaline pretreatment of rats on removal of 5-hydroxytryptamine and noradrenaline by perfused lung. Brit. J. Pharmacol. 63: 435-443, 1978.

Gillis, C.N. and Pitt, B.R.: The fate of circulating amines within the pulmonary circulation. Ann. Rev. Physiol. 44: 269-282, 1982.

Gimbrone, M.A., Aster, R.H., Cotran, R.S., Corkery, J.H., Jandl, J.H. and Folkman, J.: Preservation of vascular integrity in organs perfused in vitro with a platelet-rich medium. Nature 222: 33-36, 1969.

Given, M.B. and Longenecker, G.L.: Characteristics of serotonin uptake and release by platelets. In: The Platelets: Physiology and Pharmacology (G.L. Lonenecker, ed.). Academic Press, New York, 1985, pp. 463-479.

Glusa, E. and Markwardt, F.: Inhibition of 5-hydroxytryptamine-potentiated aggregation of human blood platelets by 5-hydroxytryptamine receptor-blocking agents. Biomed. Biochim. Acta 43: 215-220, 1984.

Goldsmith, J.C. and McCormick, J.J.: Immunologic injury to vascular endothelial cells: Effects on release of prostacyclin. Blood 63: 984-989, 1984.

Goldsmith, J.C. and Needleman, S.W.: A comparative study of thromboxane and prostacyclin release from ex vivo and cultured bovine vascular endothelium. Prostaglandins 24: 173-178, 1982.

Goldstein, I.M., Malmsten, C.L., Kindahl, H., Kaplan, H.B., Radmark, O., Samuelson, B. and Weissman, G.: Thromboxane generation by human peripheral blood polymorphonuclear leukocytes. J. Exp. Med. 148: 787-792, 1978.

Gorman, R.R.: Modulation of human platelet function by prostacyclin and thromboxane A2. Fed. Proc. 38: 83-88, 1979.

Greenwald, J.E., Wong, L.K., Rao, M., Bianchine, J.R. and Panganamala, R.V.: A study of three vasodilating agents as selective inhibitors of thromboxane A2 biosynthesis. Biochem. Biophys. Res. Commun. 84: 1112-1118, 1978.

Gryglewski, F.J., Bunting, S., Moncada, S., Flower, R.J. and Vane J.R.: Arterial walls are protected against deposition of platelet thrombi by a substance (Prostaglandin X) which they make from prostaglandin endoperoxides. Prostaglandins 12: 685-713, 1976.

Hacker, A. and Isaghulian, S.: Dietary restriction, polyamines and monocrotaline-induced pulmonary hypertension. Fed. Proc. 45: 218, 1986.

Hamberg, M., Hedqvist, P., Strandberg, K., Svensson, J. and Samuelsson, B.: Prostaglandin endoperoxides. IV. Effects on smooth muscle. Life Sci. 16: 451-462, 1975a.

Hamberg, M., Svensson, J. and Samuelsson, B.: Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides. Proc. Natl. Acad. Sci. 72: 2994-2998, 1975b.

Hamberg, M., Svensson, J., Wakabayashi, T. and Samuelsson, B.: Isolation and structure of two prostaglandin endoperoxides that cause platelet aggregation. Proc. Natl. Acad. Sci. 71: 345-349, 1974.

Hayashi, Y.: Excretion and alteration of monocrotaline in rats after a subcutaneous injection. Fed. Proc. 25: 688, 1966.

Hayashi, Y., Kato, M. and Otsuka, H.: Inhibitory effects of diet restriction on monocrotaline intoxication in rats. Toxicol. Lett. 3: 151-155, 1979.

Hayashi, Y., Kokubo, T., Takahashi, M., Furukawa, F., Otsuka, H. and Hashimoto, K.: Correlative morphological and biochemical studies on monocrotaline-induced pulmonary alterations in rats. Toxicol. Lett. 21: 65-71, 1984.

Hayashi, Y. and Lalich, J.J.: Renal and pulmonary alterations induced in rats by a single injection of monocrotaline. Proc. Soc. Exp. Biol. Med. 124: 392-396, 1967.

Heath, D.: Pulmonary hypertension due to Crotalaria spectabilis. In: The Pulmonary Circulation and Interstitial Space (A.P. Fishman and H.H. Hecht, eds.). University of Chicago Press, Chicago, 1969, pp. 305-319.

Heath, D. and Smith, P.: The electron microscopy of "fibrinoid necrosis" in pulmonary arteries. Thorax 33: 579-595, 1978.

Heby, O.: Roles of polyamines in the control of cell proliferation and differentiation. Differentiation 14: 1-20, 1981.

Hedqvist, P., Dahlen, S-E. and Bjork, J.: Pulmonary and cardiovascular pharmacology of leukotrienes. In: Leukotrienes and Prostacyclin (F. Berti, G. Folco and G.P. Velo, eds.). Plenum Press, New York, 1983, pp. 81-105.

Heffner, J.E., Shoemaker, S.A., Canham, E.M., Patel, M., McMurtry, I.F., Morris, H.G. and Repine, J.E.: Acetyl glyceryl ether phosphorycholine-stimulated human platelets cause pulmonary hypertension and edema in isolated rabbit lungs. J. Clin. Invest. 71: 351-357, 1983.

Hidaka, T., Nakano, M., Ueta, T., Komatsu, Y. and Yamamoto, M.: Increased synthesis of thromboxane A_2 by platelets from patients with Kawasaki disease. J. Pediat. 102: 94-96, 1983.

Higenbottam, T., Wells, F., Wheeldon, D. and Wallwork, J.: Long-term treatment of primary pulmonary hypertension with continuous intravenous epoprostenol (prostacyclin). Lancet 1(8385): 1046-1047, 1984.

Hill, K.R., Rhodes, K., Stafford, J.L. and Aub, R.: Liver disease in Jamaican children (serous hepatitis). West Indian Med. J. 1: 49-63, 1951.

Hilliker, K.S., Bell, T.G., Lorimer, D. and Roth, R.A.: Effects of thrombocytopenia on monocrotaline pyrrole-induced pulmonary hypertension. Am. J. Physiol. 246 (Heart Circ. Physiol. 15): H747-H753, 1984a.

Hilliker, K.S., Bell, T.G. and Roth, R.A.: Pneumotoxicity and thrombocytopenia after single injection of monocrotaline. Am. J. Physiol. 242 (Heart Circ. Physiol. 11): H573-H579, 1982.

Hilliker, K.S., Bell, T.G. and Roth, R.A.: Monocrotaline pyrrole-induced pulmonary hypertension in Fawn-hooded rats with platelet storage pool deficiency: 5-Hydroxytryptamine uptake by isolated, perfused lungs. Thromb. Haemostas. (Stuttgart) 50: 844-847, 1983a.

Hilliker, K.S., Deyo, J.A., Bell, T.G. and Roth, R.A.: Aggregation of platelets from monocrotaline pyrrole-treated rats. Thrombosis Res. 32: 325-333, 1983b.

Hilliker, K.S., Garcia, C.M. and Roth, R.A.: Effects of monocrotaline and monocrotaline pyrrole on 5-hydroxytryptamine and paraquat uptake by lung slices. Res. Comm. Chem. Pathol. Pharmacol. 40: 179-197, 1983c.

Hilliker, K.S., Imlay, M. and Roth, R.A.: Effect of monocrotaline treatment on norepinephrine removal by isolated, perfused rat lungs. Biochem. Pharmacol. 33: 2692-2695, 1984b.

Hilliker, K.S. and Roth, R.A.: Alteration of monocrotaline pyrrole-induced cardiopulmonary effects in rats by hydrallazine, dexamethasone, or sulphinpyrazone. Br. J. Pharmacol. 82: 375-380, 1984.

Hilliker, K.S. and Roth, R.A.: Increased vascular responsiveness in lungs of rats with pulmonary hypertension induced by monocrotaline pyrrole. Am. Rev. Resp. Dis. 131: 46-50, 1985a.

Hilliker, K.S. and Roth, R.A.: Injury to the isolated, perfused lung by exposure in vitro to monocrotaline pyrrole. Exp. Lung Res. 8: 201-212, 1985b.

Hislop, A. and Reid, L.: Arterial changes in Crotalaria spectabilis-induced pulmonary hypertension in rats. Brit. J. Exp. Pathol. 55: 153-163, 1974.

Holmsen, H., Day, H.J. and Stormorken, H.: The blood platelet release reaction. Scand. J. Haematol. Suppl. 8: 3-26, 1969.

Hooson, J. and Grasso, P.: Cytotoxic and carcinogenic response to monocrotaline pyrrole. J. Pathol. 118: 121-129, 1976.

Hoover, R.L., Karnovsky, M.J., Austen, K.F., Corey, E.J. and Lewis, R.A.: Leukotriene B_4 action on endothelium mediates augmented neutrophil/endothial adhesion. Proc. Natl. Acad. Sci. 81: 2191-2193, 1984.

Hsu, I.C., Allen, J.R. and Chesney, C.F.: Identification and toxicological effects of dehydroretronecine, a metabolite of monocrotaline. Proc. Soc. Exp. Biol. Med. 144: 834-838, 1973.

Hurley, J.V. and Jago, M.V.: Pulmonary oedema in rats given dehydromonocrotaline: A topographic and electron microscope study. J. Pathol. 117: 23-32, 1975.

Huxtable, R.J.: New aspects of the toxicology and pharmacology of pyrrolizidine alkaloids. Biochem. Pharmacol. 10: 159-167, 1979.

Huxtable, R.J., Ciaramitaro, D. and Eisenstein, D.: The effect of a pyrrolizidine alkaloid, monocrotaline and a pyrrole dehydroretronecine, on the biochemical functions of the pulmonary endothelium. Mol. Pharmacol. 14: 1189-1203, 1978.

Huxtable, R., Paplanus, S. and Laugharn, J.: The prevention of monocrotaline induced right ventricular hypertrophy. Chest (Suppl.) 71: 308-310, 1977.

Huzoor-Akbar, and Anwer, K.: Evidence that the rat is not an appropriate model to study the role of prostaglandins in normal or abnormal platelet aggregation. Thromb. Res. 41: 555-566, 1986.

Hyman, A.L., Mathe, A.A., Leslie, C.A., Matthews, C.C., Bennett, J.T., Spannkake, E.W. and Kadowitz, P.J.: Modification of pulmonary vascular responses to arachidonic acid by alterations in physiologic state. J. Pharmacol. Exp. Ther. 207: 388-401, 1978.

IARC: Monocrotaline Monograph on Evaluation of Carcinogenic Risk of Chemicals in Man 10: 291-302, 1976.

Ingerman-Wojenski, C., Silver, M.J., Smith, J.B. and Macarak, E.: Bovine endothelial cells in culture produce thromboxane as well as prostacyclin. J. Clin. Invest. 67: 1292-1296, 1981.

Jaffe, B.M. and Behrman, H.R.: In: Methods of Hormone Radioimmunoassay. Academic Press, New York, 1974.

Johnson, K.J. and Ward, P.A.: Acute immunologic pulmonary alveolitis. J. Clin. Invest. 54: 349-357, 1974.

Kadowitz, P.J., Gruetter, C.A., McNamara, D.B., Gorman, R.R., Spannhake, E.W. and Hyman, A.L.: Comparative effects of endoperoxide PGH_2 and an analog on the pulmonary vascular bed. J. Appl. Physiol. 42: 953-958, 1977.

Kadowitz, P.J. and Hyman, A.L.: Comparative effects of thromboxane B_2 on the canine and feline pulmonary vascular bed. J. Pharmacol. Exp. Ther. 213: 300-305, 1980.

Kajihara, H.: Electron microscopic observations of hypertrophied myocardium of rat produced by injection of monocrotaline. Acta Pathol. Jap. 20: 183-206, 1970.

Kameji, R., Otsuka, H. and Hayashi, Y.: Increase of collagen synthesis in pulmonary arteries of monocrotaline-treated rats. Experientia 36: 441-442, 1980.

Kasturi, T.E., Manchanda, S.C., Tandon, R.K., Rajani, M. and Bhatia, M.L.: Haemodynamic studies in venoocclusive disease of the liver. Brit. Heart J. 41: 594-599, 1979.

Kato, R. and Gillette, J.R.: Effect of starvation on NADPH-dependent enzymes in liver microsomes of male and female rats. J. Pharmacol. Exp. Ther. 150: 279-284, 1965.

Kay, J.M. and Heath, D.: Crotalaria spectabilis -- The Pulmonary Hypertension plant. Springfield, IL: Charles C. Thomas, 1969.

Kay, J.M., Gillund, T.D. and Heath, D.: Mast cells in the lungs of rats fed on Crotalaria spectabilis seeds. Am. J. Pathol. 51: 1031-1044, 1967a.

Kay, J.M., Harris, P. and Heath, D.: Pulmonary hypertension produced in rats by ingestion of Crotalaria spectabilis seeds. Thorax 22: 176-179, 1967b.

Kay, J.M. and Heath, D.: Observations on the pulmonary arteries and heart weight of rats fed on Crotalaria spectabilis seeds. J. Pathol. Bact. 92: 385-394, 1966.

Kay, J.M., Keane, P.M. and Suyama, K.L.: Pulmonary hypertension induced in rats by monocrotaline and chronic hypoxia is reduced by p-chlorophenylalanine. Respiration 47: 48-56, 1985.

Kay, J.M., Keane, P.M., Suyama, K.L. and Gauthier, D.: Angiotensin converting enzyme activity and evaluation of pulmonary vascular disease in rats with monocrotaline pulmonary hypertension. Thorax 37: 88-96, 1982a.

Kay, J.M., Smith, P. and Heath, D.: Electron microscopy of Crotalaria pulmonary hypertension. Thorax 24: 511-526, 1969.

Kay, J.M., Suyama, K.L. and Keane, P.M.: Failure to show decrease in small pulmonary blood vessels in rats with experimental pulmonary hypertension. Thorax 37: 927-930, 1982b.

Keane, P.M. and Kay, J.M.: Lung angiotensin converting enzyme activity in monocrotaline pulmonary hypertension (Letter). Thorax 39: 159, 1984.

Keane, P.M., Kay, J.M., Suyama, K.L., Gauthier, D. and Andrew, K.: Lung angiotensin converting enzyme activity in rats with pulmonary hypertension. Thorax 37: 198-204, 1982.

King, G.L. and Buchwald, S.: Characterization and partial purification of an endothelial cell growth factor from human platelets. J. Clin. Invest. 73: 392-396, 1984.

Kitchens, C.S. and Pendergast, J.F.: Human thrombocytopenia is associated with structural abnormalities of the endothelium that are ameliorated by glucocorticosteroid administration. Blood 67: 203-206, 1986.

Kitchens, C.S. and Weiss, L.: Ultrastructural changes of endothelium associated with thrombocytopenia. Blood 46: 567-578, 1975.

Kopolovic, R., Thrailkill, K.M., Martin, D.T., Ambose, T., Vento, M., Carey, L.C. and Cloutier, C.T.: Effects of ibuprofen on a porcine model of acute respiratory failure. J. Surg. Res. 36: 300-305, 1984.

Laduron, P.M., Janssen, P.F.M. and Leysen, J.E.: In vivo binding of (^{3}H)ketanserin on serotonin S_2-receptors in rat brain. Eur. J. Pharmacol. 81: 43-48, 1982.

Lafranconi, W.M., Duhamel, R.C., Brendel, K. and Huxtable, R.J.: Differentiation of the cardiac and pulmonary toxicity of monocrotaline, a pyrrolizidine alkaloid. Biochem. Pharmacol. 33: 191-197, 1984.

Lafranconi, W.M. and Huxtable, R.J.: Changes in angiotensin converting enzyme activity in lungs damaged by the pyrrolizidine alkaloid monocrotaline. Thorax 38: 307-309, 1983.

Lafranconi, W.M. and Huxtable, R.J.: Hepatic metabolism and pulmonary toxicity of monocrotaline using isolated perfused liver and lung. Biochem. Pharmacol. 33: 2479-2484, 1984.

Lalich, J.J. and Ehrhart, L.A.: Moncorotaline-induced pulmonary arteritis in rats. J. Atherosclerosis Res. 2: 482-492, 1962.

Lalich, J.J., Johnson, W.D., Raczniak, T.J. and Schumacher, R.C.: Fibrin thrombosis in monocrotaline pyrrole-induced cor-pulmonale in rats. Arch. Pathol. Lab. Med. 101: 69-73, 1977.

Lampagnani, M.G. and DeGaetano, G.: Comparative effects of ketanserin, a novel serotonergic receptor antagonist, on 5HT-induced shape change and 5HT uptake in rat and human platelets. Biochem. Pharmacol. 31: 3000-3002, 1982.

Langleben, D., Calvalho, A.C.A. and Reid, L.M.: The platelet thromboxane inhibitor, Dazmegrel, does not reduce monocrotaline-induced pulmonary hypertension. Am. Rev. Respir. Dis. 133: 789-791, 1986.

Langleben, D. and Reid, L.M.: Effect of methylprednisolone on monocrotaline-induced pulmonary vascular disease and right ventricular hypertrophy. Lab. Invest. 52: 298-303, 1985.

Laubscher, A. and Pletscher, A.: Shape change and uptake of 5-hydroxytryptamine in human blood platelets: Action of neuropsychotropic drugs. Life Sciences 24: 1833-1840, 1979.

Leach, C.M. and Thorburn, G.D.: A comparative study of collagen induced thromboxane release from platelets of different species: implication for human atherosclerosis models. Prostaglandins 24: 47-59, 1982.

Leffler, C.W., Mitchell, J.A. and Green, R.S.: Cardiovascular effects of leukotrienes in neonatal piglets. Circ. Res. 55: 780-787, 1984.

Leysen, J.E., Awouters, F., Kennis, L., Laduron, P.M., Vandenberk, J. and Janssen, P.A.J.: Receptor binding profile of R 41 468, a novel antagonist at 5-HT_2 receptors. Life Sciences 28: 1015-1022, 1980.

Lichey, J., Friedrich, T., Franke, J., Nigam, S., Priesnitz, M. and Oeff, K.: Pressure effects and uptake of platelet-activating factor in isolated rat lung. J. Appl. Physiol. 57: 1039-1044, 1984.

Livio, M., Villa, S. and DeGaetano, G.: Long-lasting inhibition of platelet prostaglandin but normal vascular prostacyclin generation following sulphinpyrazone administration to rats. J. Pharm. Pharmacol. 32: 718-719, 1980.

Longenecker, G.L.: Platelet arachidonic acid metabolism. In: The Platelets: Physiology and Pharmacology (G.L. Longenecker, ed.). Academic Press, New York, 1985, pp. 49-83.

Longenecker, G.L., Swift, L.A., Bowen, R.J., Beyers, B.J. and Shah, A.K.: Kinetics of ibuprofen effect on platelet and endothelial prostanoid release. Clin. Pharmacol. Ther. 37: 343-348, 1985.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275, 1951.

Macconi, K., Morgenti, G., Livio, M., Morelli, C., Cassina, G. and Remuzzi, G.: Acetyl glycerylphosphorylcholine aggregates human platelets through two distinct pathways, both dependent on arachidonic acid metabolism. Lab. Invest. 52: 159-168, 1985.

MacIntyre, D.E., Pearson, J.D. and Gordon, J.L.: Localisation and stimulation of prostacyclin production in vascular cells. Nature 271: 549-551, 1978.

Malmsten, C.: Some biological effects of prostaglandin endoperoxide analogs. Life Sci. 18: 169-176, 1976.

Malmsten, C., Hamberg, M., Svensson, J. and Samuelsson, B.: Physiologic role of an endoperoxide in human platelets: Hemostatic defect due to platelet cyclo-oxygenase deficiency. Proc. Natl. Acad. Sci. 72: 1446-1450, 1975.

Masugi, Y., Oami, H., Aihara, K., Hashimoto, K. and Hagozaki, T.: Renal and pulmonary vascular changes induced by Crotalaria spectabilis in rats. Acta Pathol. Japan 15: 407-415, 1965.

Mathews, W.R. and Murphy, R.C.: Inhibition of leukotriene biosynthesis in mastocytoma cells by diethylcarbamazine. Biochem. Pharmacol. 31: 2129-2132, 1982.

Mattocks, A.R.: Toxicity of pyrrolizidine alkaloids. Nature (London) 217: 723-728, 1968.

Mattocks, A.R.: Dihydropyrrolizidine derivatives from unsaturated pyrrolizidine alkaloids. J. Chem. Soc. C: 1155-1162, 1969.

Mattocks, A.R.: Hepatotoxic effects due to pyrrolizidine alkaloid N-oxides. Xenobiotica 1: 563-565, 1971.

Mattocks, A.R.: Acute hepatotoxicity of pyrrolic metabolites in rats dosed with pyrrolizidine alkaloids. Chem.-Biol. Interact. 5: 227-242, 1972.

Mattocks, A.R. and White, I.N.H.: Estimation of metabolites of pyrrolizidine alkaloids in animal tissues. Anal Biochem. 38: 529-535, 1970.

Mattocks, A.R. and White, I.N.H.: The conversion of pyrrolizidine alkaloids to N-oxides and to dihydropyrrolizidine derivatives by rat liver microsomes in vitro. Chem.-Biol. Interact. 3: 383-396, 1971.

Mauderly, J.L.: Bronchopulmonary lavage of small laboratory animals. Lab. Anim. Sci. 27: 255-261, 1977.

McDonald, J.W.D., Ali, M., Morgan, E., Townsend, E.R. and Cooper, J.D.: Thromboxane synthesis by sources other than platelets in association with complement-induced pulmonary leukostasis and pulmonary hypertension in sheep. Circ. Res. 52: 1-6, 1983.

McGoon, M.D. and Vanhoutte, P.M.: Aggregating platelets contract isolated canine pulmonary arteries by releasing 5-hydroxytryptamine. J. Clin. Invest. 74: 828-833, 1984.

McGregor, L. and Renaud, S.: Effect of dietary linoleic acid deficiency on platelet aggregation and phospholipid fatty acids of rats. Thromb. Res. 12: 921-927, 1978.

McIntyre, B.A. and Philp, R.B.: Effect of three nonsteroidal anti-inflammatory agents on platelet function and prostaglandin synthesis in vitro. Thromb. Res. 12: 67-77, 1977.

McLean, E.K.: Toxic actions of pyrrolizidine (Senecio) alkaloids. Pharmacol. Rev. 22: 429-483, 1970.

McLeod, A.A. and Jewitt, D.E.: Drug treatment of primary pulmonary hypertension. Drugs 31: 177-184, 1986.

McNabb, L.J. and Baldwin, K.M.: Hemodynamic and metabolic effects of exercise in Crotalaria-induced pulmonary hypertension in rats. J. Appl. Physiol: Respirat. Environ. Exercise Physiol. 57: 1829-1833, 1984.

Mehta, P., Mehta, J. and Hay, D.: Thromboxane and prostacyclin generation by intact human vessels in response to balloon catheter trauma. Prostaglandins, Leukotrienes and Med. 9: 539-548, 1982.

Menconi, M., Hahn, G. and Polgar, P.: Prostaglandin synthesis by cells comprising the calf pulmonary artery. J. Cell. Physiol. 120: 163-168, 1984.

Merkow, L. and Kleinerman, J.: An electron microscopic study of pulmonary vasculitis induced by monocrotaline. Lab. Invest. 15: 547-564, 1966.

Meyers, K.M., Seachord, C.L., Holmsen, H., Smith, J.B. and Prieur, D.J.: A dominant role of thromboxane formation in secondary aggregation of platelets. Nature 282: 331-333, 1979.

Meyrick, B., Gamble, W. and Reid, L.: Development of Crotalaria pulmonary hypertension: Hemodynamic and structural study. Am. J. Physiol. 239 (Heart Circ. Physiol. 8): H692-H702, 1980.

Meyrick, B. and Reid, L.: Development of pulmonary arterial changes in rats fed Crotalaria spectabilis. Am. J. Pathol. 94: 37-50, 1979.

Meyrick, B.O. and Reid, L.M.: Crotalaria-induced pulmonary hypertension. Uptake of ^{3}H-thymidine by the cells of the pulmonary circulation and alveolar walls. Am. J. Pathol. 106: 84-94, 1982.

Mgbodile, M.U.K., Hayes, J.R. and Campbell, T.C.: Effect of protein deficiency on the inducibility of the hepatic microsomal drug-metabolizing enzyme system II (Effect on enzyme kinetics and the electron transport system). Biochem. Pharmacol. 22: 1125-1132, 1973.

Miller, W.C., Rice, D.L., Unger, K.M. and Bradley, B.L.: Effect of PEEP on lung water content in experimental noncardiogenic pulmonary edema. Crit. Care Med. 9: 7-9, 1981.

Miranda, C.L., Reed, R.L., Cheeke, P.R. and Buhler, D.R.: Protective effects of butylated hydroxyanisole against the acute toxicity of monocrotaline in mice. Toxicol. Appl. Pharmacol. 59: 424-430, 1981.

Mohabbat, O., Shafig, Y.M., Mercao, A., Srivastave, R.N., Gholem, G.S. and Aram, G.N.: An outbreak of hepatic veno-occlusive disease in North-western Afghanistan. Lancet 269-271, 1976.

Mojarad, M., Hamasaki, Y. and Said, S.I.: Platelet-activating factor increases pulmonary microvascular permeability and induces pulmonary edema. Bull. Europ. Physiopath. Resp. 19: 253-256, 1983.

Molteni, A., Ward, W.F., Ts'ao, C.H., Port, C.D. and Solliday, N.: Monocrotaline-induced pulmonary endothelial dysfunction in rats. Proc. Soc. Exp. Biol. Med. 176: 88-94, 1984.

Molteni, A., Ward, W.F., Ts'ao, C-H. and Solliday, N.H.: Modification of monocrotaline-induced pulmonary injury by the angiotensin converting enzyme inhibitors CGS13945 and CGS16617. Fed. Proc. 45: 461, 1986.

Molteni, A., Ward, W.F., Ts'ao, C-H., Solliday, N.H. and Dunne, M.: Monocrotaline-induced pulmonary fibrosis in rats: Amelioration by captopril and penicillamine. Proc. Soc. Exp. Biol. Med. 180: 112-120, 1985.

Moncada, S., Gryglewski, R., Bunting, S. and Vane, J.R.: An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. Nature 263: 663-665, 1976.

Mooi, W. and Wagenvoort, C.A.: Decreased numbers of pulmonary blood vessels: Reality or artifact? J. Pathol. 141: 441-447, 1983.

Morel, D.R., Dargent, F., Bachmann, M., Suter, P.M. and Junod, A.F.: Pulmonary extraction of serotonin and propranolol in patients with adult respiratory distress syndrome. Am. Rev. Respir. Dis. 132: 479-484, 1985.

Morganroth, M.L., Pickett, W., Reeves, J.T. and Voelkel, N.F.: Rats raised on an essential fatty acid deficient (EFAD) diet have decreased pulmonary vascular responsiveness. Am. Rev. Respir. Dis. 131: A404, 1985.

Mullane, K.M., Bradley, G. and Moncada, S.: The interactions of platelet-derived mediators on isolated canine coronary arteries. Eur. J. Pharmacol. 84: 115-118, 1982.

Nawroth, P.P., Stern, D.M. and Dietrich, M.: Thromboxane production by perturbed bovine aortic endothelial cells in culture. Blut 50: 7-11, 1985.

Nawroth, P.P., Stern, D.M., Kaplan, K.L. and Nossel, H.L.: Prostacyclin production by perturbed bovine aortic endothelial cells in culture. Blood 64: 801-806, 1984.

Needleman, P., Moncada, S., Bunting, S., Vane, J.R., Hamberg, M. and Samuelsson, B.: Identification of an enzyme in platelet microsomes which generates thromboxane A_2 from prostaglandin endoperoxides. Nature 261: 558-560, 1976.

Nishizawa, E.E., Williams, D.J. and Connell, C.L.: Arachidonate induced aggregation of rat platelets may not require prostaglandin endoperoxides or thromboxane A_2. Thromb. Res. 30: 289-296, 1983.

Ody, C., Seillan, C. and Russo-Marie, F.: 6-Ketoprostaglandin $F_{1\alpha}$, prostaglandins E_2, $F_{2\alpha}$ and thromboxane B_2 production by endothelial cells, smooth muscle cells, and fibroblasts cultured from piglet aorta. Biochim. Biophys. Acta 712: 103-110, 1982.

Ogunbiyi, P.O. and Eyre, P.: Tryptaminergic receptors in the bovine pulmonary vasculature: Effects of ketanserin. Res. Comm. Chem. Pathol. Pharmacol. 44: 31-55, 1984.

Olson, J.W., Altiere, R.J. and Gillespie, M.N.: Prolonged activation of rat ornithine decarboxylase in monocrotaline induced pulmonary hypertension. Biochem. Pharmacol. 33: 3633-3637, 1984a.

Olson, J.W., Atkinson, J.E., Hacker, A.D., Altiere, R.J. and Gillespie, M.N.: Suppression of polyamine biosynthesis prevents monocrotaline-induced pulmonary edema and arterial medial thickening. Toxicol. Appl. Pharmacol. 81: 91-99, 1985.

Olson, J.W., Hacker, A.D., Altiere, R.J. and Gillespie, M.N.: Polyamines and the development of monocrotaline-induced pulmonary hypertension. Am. J. Physiol. 247: H682-H685, 1984b.

Palevsky, H.I. and Fishman, A.P.: Vasodilator therapy for primary pulmonary hypertension. Ann. Rev. Med. 36: 563-578, 1985.

Parise, L.V., Venton, D.L. and LeBreton, G.C.: Arachidonic acid-induced platelet aggregation is mediated by a thromboxane A_2/prostaglandin H_2 receptor interaction. J. Pharmacol. Exp. Ther. 228: 240-244, 1984.

Parry, M.J., Randall, M.J., Hawkeswood, E., Cross, P.E. and Dickinson, R.P.: Enhanced production of prostacyclin in blood after treatment with selective thromboxane synthetase inhibitor, UK-38,485. Br. J. Pharmacol. 77: 547P, 1982.

Peroutka, S.J. and Snyder, S.H.: Multiple serotonin receptors: Differential binding of (^{3}H)5-hydroxytryptamine, (^{3}H)lysergic acid diethylamide and (^{3}H)spiroperidol. Mol. Pharmacol. 16: 687-699, 1979.

Plestina, R. and Stoner, H.B.: Pulmonary oedema in rats given monocrotaline pyrrole. J. Pathol. 106: 235-249, 1972.

Raczniak, T.J., Chesney, C.F. and Allen, J.R.: Ultrastructure of the right ventricle after monocrotaline-induced cor pulmonale in the nonhuman primate (Macaca arctoides). Exp. Mol. Pathol. 28: 107-118, 1978.

Raczniak, T.J., Shumaker, R.C., Allen, J.R., Will, J.A. and Lalich, J.J.: Pathophysiology of dehydromonocrotaline-induced pulmonary fibrosis in the beagle. Resp. 37: 252-260, 1979.

Ratliff, N.B., Gerrard, J.M. and White, J.G.: Platelet-leukocyte interactions following arterial endothelial injury. Am. J. Pathol. 96: 567-580, 1979.

Ratnoff, O.D. and Mirick, G.S.: Influence of sex upon the lethal effects of an hepatotoxic alkaloid, monocrotaline. Bull. Johns Hopkins Hosp. 84: 507-525, 1949.

Raymond, S.L. and Dodds, W.J.: Characterization of the fawn-hooded rats as a model for hemostatic studies. Thromb. Diath. Haemorrh. 33: 361-369, 1975.

Reid, L.M.: Structure and function in pulmonary hypertension. Chest 89: 279-288, 1986.

Rickaby, D.A., Dawson, C.A. and Maron, M.B.: Pulmonary inactivation of serotonin and site of serotonin pulmonary vasoconstriction. J. Appl. Physiol. 48: 606-612, 1980.

Rose, C.L., Fink, R.D., Harris, P.N. and Chen, K.K.: The effect of hepatotoxic alkaloids on the prothrombin time of rats. J. Pharmacol. Exp. Ther. 83: 265-269, 1945.

Rose, A.L., Gardner, C.A., McConnell, D. and Bull, B.: Field and experimental investigation of "walk-about" disease of horses (Kimberly horse disease) in northern Australia: Crotalaria poisoning in horses. Part II. Aust. Vet. J. 33: 49-62, 1957.

Roth, R.A.: Effects of pneumotoxicants on lactate dehydrogenase activity in airways of rats. Toxicol. Appl. Pharmacol. 57: 69-78, 1981.

Roth, R.A.: Biochemistry, physiology and drug metabolism -- Implications regarding the role of the lungs in drug disposition. Clin. Physiol. Biochem. 3: 66-79, 1985.

Roth, R.A., Dotzlaf, L.A., Baranyi, B., Kuo, C-H. and Hook, J.B.: Effect of monocrotaline ingestion on liver, kidney and lung of rats. Toxicol. Appl. Pharmacol. 60: 193-203, 1981.

Roum, J.H., McNabb, L.J. and Baldwin, K.M.: Effect of prostaglandin E_1 on Crotalaria-induced pulmonary hypertension. Clin. Res. 31: 74A, 1983.

Roy, A.J. and Djerassi, I.: Effects of platelet transfusions: Plug formation and maintenance of vascular integrity. Proc. Soc. Exp. Biol. Med. 139: 137-142, 1972.

Rubin, D.B., Drab, E.A., Ts'ao C-H., Gardner, D. and Ward, W.F.: Prostacyclin synthesis in irradiated endothelial cells cultured from bovine aorta. J. Appl. Physiol. 58: 592-597, 1985.

Ryan, U.S. and Ryan, J.W.: Cell biology of pulmonary endothelium. Circulation 70(Suppl. III): III-46 - III-62, 1984.

Saldeen, P. and Saldeen, T.: 6-Keto-prostaglandin $F_{1\alpha}$/thromboxane B2 ratio in vascular and lung tissue. Thromb. Res. 30: 643-650, 1983.

Salzman, P.M., Salmon, J.A. and Moncada, S.: Prostacyclin and thromboxane A_2 synthesis by rabbit pulmonary artery. J. Pharmacol. Exp. Ther. 215: 240-247, 1980.

Schoenthal, R. and Head, M.A.: Pathological changes in rats as a result of treatment with monocrotaline. Brit. J. Cancer 9: 229-237, 1955.

Schoenthal, R., Head, M.A. and Peacock, P.R.: Senecio alkaloids: Primary liver tumors in rats, as a result of treatment with (1) a mixture of alkaloids from S. jacobaea Lin. (2) retrorsine (3) isatidine. Br. J. Cancer 8: 458-462, 1954.

Seeger, W., Bauer, M. and Bhakdi, S.: Staphylococcal α-toxin elicits hypertension in isolated rabbit lungs. J. Clin. Invest. 74: 849-858, 1984.

Shannon, N.J., Gunnet, J.W. and Moore, K.E.: Comparison of biochemical indices of 5-hydroxytryptaminergic neuronal activity following electrical stimulation of the dorsal raphe nucleus. J. Neurochem., in press, 1986.

Shepro, D., Sweetman, H.E. and Hechtman, H.B.: Experimental thrombocytopenia and capillary ultrastructure. Blood 56: 937-939, 1980.

Shoemaker, S.A., Heffner, J.E., Canhman, E.M., Tate, R.M., Morris, H.G., McMurtry, I.F. and Repine, J.E.: Staphylococcus aureus and human platelets cause pulmonary hypertension and thromboxane generation in isolated saline-perfused rabbit lungs. Am. Rev. Resp. Dis. 129: 92-95, 1984.

Simpson, C.F., Waldroup, P.W. and Harms, R.H.: Pathologic changes associated with feeding various levels of Crotalaria spectabilis seed to poultry. J. Am. Vet. Med. Assoc. 142: 264-271, 1963.

Sirois, P., Chagnon, M., Borgeat, P. and Vallerand, P.: Role of cyclooxygenase products in the lung action of leukotrienes A_4, B_4, C_4, D_4 and E_4. Pharmacology 31: 225-236, 1985.

Snow, R.L., Davies, P., Pontoppidan, H., Zapol, W.M. and Reid, L.: Pulmonary vascular remodelling in adult respiratory distress syndrome. Am. Rev. Resp. Dis. 126: 887-892, 1982.

Snyder, S.P.: Livestock losses due to tansy ragwort poisoning. Oregon Agrirecord 255: 3-4, 1972.

Sole, M.J., Drobac, M., Schwartz, L., Hussain, M.N. and Vaughan-Neil, F.F.: The extraction of circulating catecholamines by the lungs in normal men and in patients with pulmonary hypertension. Circulation 60: 160-163, 1979.

Steel, R.G.D. and Torrie, J.H.: Principles and Procedures of Statistics, A Biometrical Approach (2nd Ed.). New York: McGraw-Hill, 1980.

Steinberg, H. and Das, D.K.: Competition between platelets and lung for 5-hydroxytryptamine uptake. Exp. Lung Res. 1: 121-130, 1980.

Stenmark, K.R., Morganroth, M.L., Remigio, L.K., Voelkel, N.F., Murphy, R.C., Henson, P.M., Mathias, M.M. and Reeves, J.T.: Alveolar inflammation and arachidonate metabolism in monocrotaline-induced pulmonary hypertension. Am. J. Physiol. 248: H859-H866, 1985.

Stillman, A.E., Huxtable, R., Consroe, P., Kohnen, P. and Smith, S.: Hepatic venoocclusive disease due to pyrrolizidine poisoning in Arizona. Gastroenterology 73: 349-352, 1977.

Stinger, R.B., Iacopino, V.J., Alter, L, Fitzpatrick, T.M., Rose, J.C. and Kot, P.A.: Catheterization of the pulmonary artery in the closed-chest rat. J. Appl. Physiol. 51: 1047-1050, 1981.

Stuart, V.L. and Bras, G.: Veno-occlusive disease of the liver. Quart. J. Med. 26: 291-315, 1957.

Sugita, T., Hyers, T.M., Dauber, I.M., Wagner, W.W., McMurtry, J.F. and Reeves, J.T.: Lung vessel leak precedes right ventricular hypertrophy in monocrotaline-treated rats. J. Appl. Physiol.: Respirat. Environ. Exercise Physiol. 54: 371-374, 1983a.

Sugita, T., Stenmark, K.R., Wagner, W.W., Jr., Henson, P., Henson, J., Hyers, T.M. and Reeves, J.T.: Abnormal alveolar cells in monocrotaline induced pulmonary hypertension. Exp. Lung Res. 5: 201-215, 1983b.

Suzuki, H. and Twarog, B.M.: Membrane properties of smooth muscle cells in pulmonary hypertensive rats. Am. J. Physiol. 242: H907-H915, 1982.

Svensson, J., Strandberg, K., Tuvemo, T. and Hamberg, M.: Thromboxane A_2: Effects on airway and vascular smooth muscle. Prostaglandins 14: 425-436, 1977.

Takeoka, O., Angevine, D.M. and Lalich, J.J.: Stimulation of mast cells in rats fed various chemicals. Am. J. Pathol. 40: 545-554, 1962.

Talvenheimo, J., Nelson, P.J. and Rudnick, G.: Mechanism of imipramine inhibition of platelet 5-hydroxytryptamine transport. J. Biol. Chem. 245: 6431-6435, 1979.

Tandon, B.N., Tandon, R.K., Tandon, J.D., Narndranthan, M. and Joshi, Y.K.: An epidemic of veno-occlussive disease of liver in central India. Lancet 271-272, 1976.

Tateson, J.E., Moncada, S. and Vane, J.R.: Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets. Prostaglandins 13: 389-397, 1977.

Thomas, E.F.: The toxicity of certain species of Crotalaria seed for the chicken, quail, turkey and dove. J. Am. Vet. Med. Assoc. 85: 617-622, 1934.

Tranzer, J.P., DaPrada, M. and Pletscher, A.: Ultrastructural localisation of 5-hydroxytryptamine in blood platelets. Nature 212: 1574-1575, 1966.

Tuchweber, B., Kovaks, K., Jago, M.V. and Beaulieu, T.: Effect of steroidal and non-steroidal microsomal enzyme inducers on the hepatotoxicity of the pyrrolizidine alkaloids in rats. Res. Comm. Chem. Pathol. Pharmacol. 7: 459-480, 1974.

Tucker, A., Bryant, S.E., Frost, H.H. and Migally, N.: Chemical sympathectomy and serotonin inhibition reduce monocrotaline-induced right ventricular hypertrophy in rats. Can. J. Physiol. Pharmacol. 61: 356-362, 1982.

Tucker, A. and Rodeghero, P.T.: Vascular responsiveness in isolated rat lungs is inversely related to blood flow. Respiration 42: 228-232, 1981.

Turner, J.H. and Lalich, J.J.: Experimental cor pulmonale in the rat. Arch. Pathol. 79: 409-418, 1965.

Vaage, J., Bø, G. and Hognestad, J.: Pulmonary responses to intravascular platelet aggregation in the cat. Acta Physiol. Scand. 92: 546-556, 1974.

Vaage, J., Nicolaysen, G. and Waaler, B.A.: Aggregation of blood platelets and increased hydraulic conductivity of pulmonary exchange vessels. Acta Physiol. Scand. 98: 175-184, 1976.

Valdivia, E., Lalich, J.J., Hayashi, Y. and Sonnand, J.: Alterations in pulmonary alveoli after a single injection of monocrotaline. Arch. Pathol. 84: 64-76, 1967.

Van Horn, D.L. and Johnson, S.A.: The mechanism of thrombocytopenic bleeding. Am. J. Clin. Pathol. 46: 204-213, 1966.

Van Nueten, J.M., Janssen, P.A.J., deRidder, W. and Vanhoutte, P.M.: Interaction between 5-hydroxytryptamine and other vasoconstrictor substances in the isolated femoral artery of the rabbit: Effect of ketanserin (R 41 468). Eur. J. Pharmacol. 77: 281-287, 1982.

Van Nueten, J.M., Xhonneux, R., Vanhoutte, P.M. and Janssen, P.A.J.: Vascular activity of ketanserin (R 41 468), a selective 5-HT_2 receptor antagonist. Arch. int. Pharmacol. Ther. 250: 328-329, 1981.

Vincent, J.-L., Degaute, J.-P., Domb, M., Simon, P., Berre, J. and Vandesteene, A.: Ketanserin, a serotonin antagonist: Administration in patients with acute respiratory failure. Chest 85: 510-513, 1984.

Voelkel, N. and Reeves, J.T.: Primary pulmonary hypertension. In Pulmonary Vascular Diseases (Moser, K.M., ed.), Vol. 14, p. 573. New York: Marcel Dekker, 1979.

Walcott, G., Burchell, H.B. and Brown, A.L., Jr.: Primary pulmonary hypertension. Am. J. Med. 49: 70-79, 1970.

Watanabe, S. and Ogata, T.: Clinical and experimental study upon primary pulmonary hypertension. Japan. Circ. J. 40: 603-610, 1976.

Weiss, H.J.: Platelets: Pathophysiology and Antiplatelet Drug Therapy. Alan R. Liss, New York, 1982.

Weksler, B.B., Marcus, A.J. and Jaffe, E.A.: Synthesis of prostaglandin I_2 (prostacyclin) by cultured human and bovine endothelial cells. Proc. Natl. Acad. Sci. 74: 3922-3926, 1977.

Werchan, P.M., Gregory, T.J., Summer, W.R. and McDonough, K.H.: In vitro right ventricular function in monocrotaline induced hypertrophy. Fed. Proc. 45: 776, 1986.

White, I.N.H. and Mattocks, A.R.: Some factors affecting the conversion of pyrrolizidine alkaloids to N-oxides and to pyrrolic derivatives in vitro. Xenobiotica 1: 503-505, 1971.

White, M.K., Shepro, D. and Hechtman, H.B.: Pulmonary function and platelet-lung interaction. J. Appl. Physiol. 34: 697-703, 1973.

Whorton, A.R., Montgomery, M.E. and Kent, R.S.: Effect of hydrogen peroxide on prostaglandin production and cellular integrity in cultured porcine aortic endothelial cells. J. Clin. Invest. 76: 295-302, 1985.