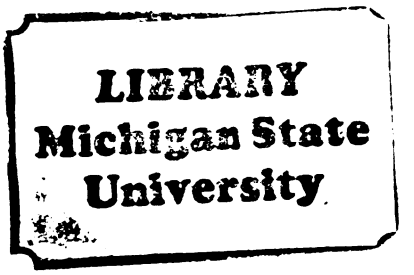

This is to certify that the

dissertation entitled

The Role of Platelets in Monocrotaline-Induced Pulmonary Hypertension

presented by

Katherine Shea Hilliker

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Pharmacology & Toxicology

Robert A. Roth
Major professor

Date March 25, 1983

THE ROLE OF PLATELETS IN MONOCROTALINE-INDUCED PULMONARY HYPERTENSION

By

Katherine Shea Hilliker

A DISSERTATION

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ABSTRACT

The Role of Platelets in Monocrotaline-Induced Pulmonary Hypertension

By

Katherine Shea Hilliker

Monocrotaline is a natural product which causes pulmonary hypertension (PH) in rats. Because of similarities in the pathology, MCT-induced PH has been suggested as a model for human primary PH. MCT toxicity requires metabolism by the liver to a toxic pyrrole, MCTP. Two possible mechanisms for the lung injury were examined: (1) that the toxic metabolite MCTP damages the endothelial cells of the lung vasculature directly and/or (2) that MCTP affects an element of the blood which then damages the lung. The platelet was chosen for study because preliminary data indicated that MCT decreases circulating platelet counts and results in platelet-containing thrombi in the lung.

MCTP caused edema and cellular damage in isolated, perfused lungs (IPL) exposed in vitro but did not affect 5-hydroxytryptamine (5HT) uptake after such short-term exposure of either the IPL or lung slices. Thus, MCTP can injure the pulmonary vasculature directly but this injury may not account for all of the effects of MCTP on the lung.

Induction of thrombocytopenia for the first two days after MCTP injection did not alter right ventricular hypertrophy (RVH) suggesting that the platelet was not involved in initiation of the lung injury. Reduction of the platelet count at later times during the experimental period did reduce the RVH, indicating that the platelet is involved in the development of the PH. The possibility of a serotonergic mechanism was investigated since 5HT is released by platelets and is a vasoconstrictor. Isolated lungs from MCTP-treated rats showed increased pressor responses to 5HT. MCT or MCTP treatment of rats also decreased the ability of the IPL to remove circulating 5HT, which might cause increased 5HT concentrations in the blood. Platelet uptake and release of 5HT probably does not play a major role since fawn-hooded rats, a strain with a congenital defect in these functions, showed no protection from MCTP-induced RVH. Likewise, metergoline, a 5HT receptor antagonist, did not protect rats from RVH. The platelet may be involved in the development of PH by non-serotonergic mechanisms. Treatment with hydralazine, dexamethasone or sulfinpyrazone reduced the RVH observed after MCTP injection, suggesting that platelet prostaglandin production could be important.

For Barbara and Jack,

without whose love, support and encouragement

I would never have come this far

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AA	Arachidonic acid
ACE	Angiotensin converting enzyme
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ASA	Acetylsalicylic acid
AII	Angiotensin II
BK	Bradykinin
BW	Body weight
CS	Control serum
DMF	N,N-Dimethylformamide
DX	Dexamethasone
FH	Fawn-hooded
GPT	Glutamic pyruvic transaminase
HD	Hydralazine
5HT	5-Hydroxytryptamine
IPL	Isolated perfused lung
LDH	Lactate dehydrogenase
lsd	Least significant difference
MCT	Monocrotaline
MCTP	Monocrotaline pyrrole (dehydromonocrotaline)
MTG	Metergoline
NE	Norepinephrine
PAF	Platelet activating factor
PAS	Anti-platelet serum
PDGF	Platelet derived growth factor
PGI_2	Prostacyclin
PH	Pulmonary hypertension
PPH	Primary pulmonary hypertension

PQ	Paraquat
PRP	Platelet rich plasma
PVR	Pulmonary vascular resistance
RTP	Retronecine pyrrole (dehydroretronecine)
RV/(LV+S)	Right ventricle to left ventricle plus septum weight ratio
RVH	Right ventricular hypertrophy
Sal	Saline
SD	Sprague-Dawley
SP	Sulfinpyrazone
TLC	Thin-layer chromatography
TXA_2	Thromboxane
Veh	Vehicle

INTRODUCTION

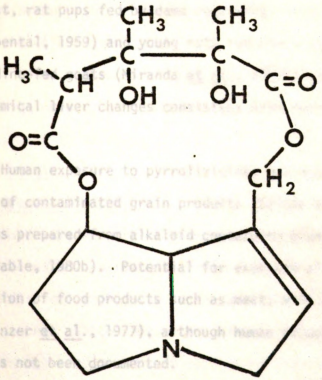
A. Pyrrolizidine Alkaloids

1. General

Monocrotaline (MCT), is a natural product found in the leaves and foliage of plants of the genus Crotalaria (Neal et al., 1935; Adams and Rogers, 1939). The common source is Crotalaria spectabilis or rattlebox. Chemically, MCT is a pyrrolizidine alkaloid, a macrocyclic diester with a pyrrolizidine nucleus. The structure of MCT is illustrated in Figure 1.

The pyrrolizidine alkaloids are widely distributed, 150 different alkaloids having been identified in 41 genera of eight plant families (Huxtable, 1979; Bull et al., 1968). Pyrrolizidine-containing plants have been recognized as toxic to animals and humans since the early 1940's (Bull et al., 1968; McLean, 1970). In the United States, the genera Crotalaria and Senecio pose the largest problems. Crotalaria spectabilis is widely distributed in the southeastern United States, while contamination of fields by Senecio jacobaea is widespread in some parts of the Pacific Northwest and livestock poisonings there are rapidly reaching epidemic proportions (Huxtable, 1980a).

Livestock are exposed to pyrrolizidine alkaloids primarily by grazing. Pyrrolizidines are also secreted into the milk of lactating animals (Dickinson et al., 1976; Dickinson, 1980; Miranda et al.,



MONOCROTALINE

Figure 1. Structure of monocrotaline

1981a), providing another possible route of exposure. The risk of intoxication by this route is unclear at present. Goats and calves fed by dams on a pyrrolizidine-containing diet have shown no evidence of liver damage or other toxicity, although the milk they received contained pyrrolizidines (Dickinson, 1980; Dickinson et al., 1976). In contrast, rat pups fed by dams receiving a pyrrolizidine-containing diet (Schoental, 1959) and young rats fed diets containing milk from pyrrolizidine-fed goats (Miranda et al., 1981a) showed histological and biochemical liver changes consistent with pyrrolizidine intoxication.

Human exposure to pyrrolizidines has occurred primarily by ingestion of contaminated grain products (Selzer and Parker, 1951) or herbal teas prepared from alkaloid containing plants (Stuart and Bras, 1957; Huxtable, 1980b). Potential for exposure also exists in the contamination of food products such as meat, milk or honey (Huxtable, 1980a; Deinzer et al., 1977), although human poisoning by these sources has not been documented.

The toxicity of the pyrrolizidine alkaloids is not limited to a particular organ or organ system. The most widely studied effects are those on the liver and the cardiopulmonary system (see below), but a number of other organs may also be affected. Monocrotaline damages the kidney (Hayashi and Lalich, 1967; Allen and Carstens, 1970; McGrath et al., 1975; Roth et al., 1981) as well as the liver and lung. Other pyrrolizidines affect different organs. Fulvine damages the pancreas (Putzke and Persaud, 1976) in addition to lung and liver. Lasiocarpine causes severe atrophy of the intestinal lining (Hooper, 1975).

Pyrrolizidine alkaloid toxicity also lacks species specificity. Virtually all species examined exhibit some type of adverse response to pyrrolizidines under the appropriate conditions. Species exhibiting toxicity after exposure to Senecio- and/or Crotalaria-derived pyrrolizidine alkaloids include man (McLean, 1970; Huxtable, 1980b), non-human primates (Chesney and Allen, 1973a), horses (Rose et al., 1957), cattle (Cushny, 1911), swine (McGrath et al., 1975), goats (Dickinson, 1980), chickens (Allen et al., 1960), turkeys (Allen et al., 1963), rainbow trout (Hendricks et al., 1981), dogs (Miller et al., 1978), mice (Miranda et al., 1981b) and rats (Schoental and Head, 1955; Roth et al., 1981).

2. Hepatic Toxicity

Most of the early research done on pyrrolizidine alkaloid toxicity focused on hepatic damage since that was the most commonly identified clinical problem in humans and livestock. In humans, the syndrome has been called veno-occlusive disease (Bras et al., 1954) and has been observed in African populations exposed to bread made from Senecio-contaminated grain (Selzer and Parker, 1951); in Jamaica and Africa where "bush teas" derived from various Crotalaria species were a popular home remedy (Stuart and Bras, 1957; McLean, 1970); and in patients in Arizona consuming herbal remedies containing Senecio alkaloids (Huxtable, 1980b). Veno-occlusive disease is characterized by occlusion of the central veins of the hepatic circulation. The occlusion, depending on the stage of the disease, may be due to swelling of the vascular intima, organized thrombi, and/or connective tissue containing collagen and elastic fibers (Bras et al., 1954).

Sinusoidal congestion, centrilobular necrosis and non-portal fibrosis also occur (Bras et al., 1954; Stuart and Bras, 1957).

Administration of Senecio or Crotalaria plant tissue or extracts has been shown to result in hepatic veno-occlusive disease in horses (Hill and Martin, 1958), cows (Bras et al., 1957), monkeys (Allen and Carstens, 1968), and rats (McLean et al., 1964). Veno-occlusive changes are seen less commonly in experimental situations than in a clinical setting, however. McLean (1970) suggests that venous occlusion may be a consequence of sudden large insults rather than the low level chronic exposure generally used for experimental purposes. Megalocytosis, centrilobular necrosis, fatty degeneration and hyperemia are the major histological changes in the liver reported in rats (Schoental and Head, 1955; Miranda et al., 1980b).

Functional evidence of liver damage in experimental animals has also been reported. Serum glutamic pyruvate transaminase levels were elevated in rats treated with fulvine (McLean et al., 1964) or monocrotaline (Roth et al., 1981). Significant lengthening of the prothrombin time after a variety of pyrrolizidine alkaloids was noted by Rose and coworkers (1945). Downward shifts in the albumin:globulin ratio were observed in rats (Miranda et al., 1980b) and turkeys (Allen et al., 1963). Biliary excretion of indocyanine green was decreased in rats after acute or chronic administration of monocrotaline (Roth et al., 1981). Alterations in drug metabolizing ability also have been reported in rats after ingestion of Senecio jacobaea (Miranda et al., 1980a).

3. Pulmonary Effects

Many pyrrolizidine alkaloids cause pulmonary toxicity (Bull et al., 1968; McLean, 1970). Of those that do, the most thoroughly studied is monocrotaline. Schoental and Head (1955) were the first to report consistent changes in the lung after exposure of rats to MCT. They noted increased size and congestion of the lungs. Histologically, alveolar hemorrhage, endothelial proliferation, and congested and dilated blood vessels were observed. These changes occurred after topical, oral or parenteral administration of MCT. The first experiments designed to examine the pulmonary effects systematically were performed by Lalich and Merkow (1961). After feeding several doses of ground Crotalaria spectabilis seed in rat chow, the predominant changes observed in the lung were pulmonary edema and alveolar hemorrhage. Pulmonary arteritis, associated with medial and periarterial infiltration of leukocytes, was also seen. To demonstrate that the pulmonary effects were due to monocrotaline in the Crotalaria spectabilis seeds and not to other alkaloids or decomposition products, rats were fed either MCT, alcohol-extracted Crotalaria seeds, or MCT decomposition products (Lalich and Ehrhart, 1962). Only MCT caused any toxicity and it mimicked the pulmonary changes observed in the previous study.

Many studies have since been published which deal with the pulmonary effects of Crotalaria or of MCT. Emphasis on the occurrence and severity of the pulmonary edema, arteritis and alveolar hemorrhage varies among investigators and with the dose, route of administration and chemical form of MCT. A comprehensive description of the pulmonary effects attributed to Crotalaria/MCT follows.

a. Gross changes. Pyrrolizidine-treated lungs were enlarged, edematous, and congested to varying degrees (Schoental and Head, 1955; Lalich and Merkow, 1961). The wet and dry weights of the lung were increased (Lalich and Merkow, 1961; Huxtable et al., 1978; Roth et al., 1981). Petechial hemorrhage and atelectatic regions were also observed on the surface of the lungs (Turner and Lalich, 1965; Merkow and Kleinerman, 1966).

b. Microscopic changes. A predominant change in the pulmonary vasculature was medial thickening of the pulmonary trunk, small arteries and arterioles. These changes have been reported by a number of investigators (Turner and Lalich, 1965; Kay and Heath, 1966; Hayashi et al., 1967; Kay et al., 1967; Meyrick and Reid, 1979). In addition to thickening of normally muscular vessels, careful morphometric analysis has demonstrated that the smooth muscle coat in MCT-treated rats extends into previously non-muscular arterioles (Meyrick and Reid, 1979; Meyrick et al., 1980). Furthermore, the medial thickening was a result of both hypertrophy and hyperplasia of the smooth muscle cells, as indicated by ^{3}H-thymidine incorporation (Meyrick and Reid, 1982).

The pulmonary arteritis, described as a common observation in experiments from Lalich's laboratory, was not observed uniformly or even frequently by other investigators. In fact, it was often noted explicitly that none or only a few rats showed the pulmonary arteritis described by Lalich (Kay and Heath, 1966; Kay et al., 1967; Meyrick and Reid, 1979; Meyrick et al., 1980). The discrepancy may simply reflect distinction in terminology. Most recent reports

describe inflammatory changes near pulmonary vessels: mononuclear cell infiltration of the periarterial region, fibrinous exudate in the alveoli, and edema in the alveolar wall. The changes are comparable to those shown and described by Lalich et al. as pulmonary arteritis. Terminology aside, inflammatory changes were commonly seen in the vicinity of the pulmonary vasculature after Crotalaria/MCT exposure. Increased numbers of mast cells in the alveolar walls (Turner and Lalich, 1965; Takeoka et al., 1962; Valdivia et al., 1967) and enlarged macrophages in the alveoli (Kay and Heath, 1966) also have been observed.

Another common finding was the presence of amorphous thrombi or fibrinoid occlusions in lumina of pulmonary arteries, arterioles, and capillaries (Turner and Lalich, 1965; Lalich and Ehrhart, 1962; Kay and Heath, 1966). Platelet-containing thrombi also were reported (Merkow and Kleinerman, 1966; Valdivia et al., 1967; Smith and Heath, 1978; Meyrick and Reid, 1982).

To understand changes in the pulmonary arterial bed, Meyrick and coworkers (Meyrick and Reid, 1979; Meyrick et al., 1980) filled the arterial side of the vasculature with radioopaque contrast medium and gelatin, under conditions designed to fill all arterial vessels down to but not including the capillary bed. Subsequent arteriograms of the filled lungs suggested a decrease in the number of pulmonary vessels less than 200 μm in diameter. This was confirmed by counting the number of vessels and alveoli in an area of known dimensions and constructing a ratio. The number of arteries decreased but the number of alveoli was unchanged. This suggests that the smaller arteries had decreased or occluded lumina.

Pulmonary veins were also affected by MCT. An early persistent change described by Smith and Heath (1978) after feeding Crotalaria seeds was smooth muscle cell evagination, suggestive of acute venoconstriction. This type of change had previously been described after chronic fulvine (isolated from Crotalaria fulva) administration or acute histamine vasoconstriction (Dingemans and Wagenvoordt, 1976). Will (1981) suggests that pulmonary veins also hypertrophy after MCT injection.

An occasional observation in MCT-treated lungs was hyperplasia of bronchial and bronchiolar epithelium (Lalich and Merkow, 1961; Lalich and Ehrhart, 1962; Turner and Lalich, 1965; Kay and Heath, 1966; Merkow and Kleinerman, 1966). The incidence reported in these studies varied from 8-35% of the treated rats. Merkow and Kleinerman (1966) observed such epithelial hyperplasia only in areas of bronchopneumonia in the lungs. It was noted in the earlier studies that many of the rats showed signs of bronchopneumonia. It is possible that the bronchiolar hyperplasia is not an effect of MCT but rather a consequence of the underlying bronchopneumonia magnified by the stress of MCT treatment.

c. Ultrastructural Changes. After MCT or Crotalaria seed administration, the capillary endothelial cells swell and protrude into the vessel lumen. Increased numbers of cytoplasmic organelles and pinocytotic vesicles and enlarged nuclei were also characteristic changes (Merkow and Kleinerman, 1966; Kay et al., 1969).

The smooth muscle cells of treated rats exhibited increased amounts of mitochondria, rough endoplasmic reticulum and Golgi complexes. Cytoplasmic lipid droplets were also increased

(Merkow and Kleinerman, 1966). Alveolar Type II cells had larger lamellar bodies, mitochondria and nuclei. The cytoplasm contained many vacuoles. Many Type II cells appeared to be free in the alveoli (Kay et al., 1969). There was a significant increase in the interstitial space between the capillary endothelial cells and alveolar epithelial cells. This space was filled by an electron-dense, amorphous material (Merkow and Kleinerman, 1966; Kay et al., 1969).

d. Cardiopulmonary effects. Focal myocarditis after Crotalaria seed ingestion was reported by Lalich and Merkow in 1961 but recognition of a hypertrophic effect on the right ventricle was not published until later (Turner and Lalich, 1965), when an increase in the total heart weight and right ventricular size were reported. Subsequently, many investigators have demonstrated an increase in the relative and/or absolute right ventricle weight. While right ventricular hypertrophy was presumed to reflect increased pulmonary vascular resistance or pressure (Fulton et al., 1952), this was not demonstrated for Crotalaria-exposed lungs until 1969 (Carillo and Aviado, 1969), when increased right ventricular pressures were measured in treated rats. Correlations between medial thickness of the pulmonary vessels, right ventricular hypertrophy, right ventricular pressure and pulmonary artery pressure have been reported (Kay and Heath, 1966; Meyrick et al., 1980; Ghodsi and Will, 1981). Pulmonary vascular resistance (PVR) was measured at various times after starting to feed ground Crotalaria seeds (Meyrick et al., 1980). At times when pulmonary arterial pressure was elevated, so was the PVR. It is clear, then, that Crotalaria/MCT exposure results in pulmonary hypertension in rats.

e. Time course of cardiopulmonary effects. Pulmonary changes have been observed by electron microscopy as early as 4 hours after an injection of 60 mg MCT/kg (Valdivia et al., 1967). At this time there are focal areas of interstitial alveolar edema, occasional thrombi in small vessels, and an increase in the number of mast cells. Twenty-four hours after injection, these changes are more pronounced and in addition, endothelial cells and interstitial cells have swollen and elastic tissue has begun to lyse. The permeability of the capillaries to Thorotrast^{R} was increased at 48 hr and all changes became more pronounced with time over the three week duration of the study. There is a lag between the early changes and the onset of pulmonary hypertension. Medial hypertrophy was seen 7 days after a single dose of MCT but increases in right heart weight and pulmonary arterial pressure were not seen until 2 weeks after treatment (Ghodsi and Will, 1981). Intermediate time points were not examined in this study. Meyrick and Reid (1979) observed no medial hypertrophy at 3 days, saw a trend toward hypertrophy at 7 days but could not demonstrate statistical significance until 10 days after commencing treatment. Unfortunately, this group of investigators administered MCT in the form of ground Crotalaria spectabilis seeds in the diet, making direct comparison with results of Ghodsi and Will (1981) difficult.

f. Biochemical and metabolic functional changes in the lung. In addition to its role in gas exchange, the lung performs a variety of non-respiratory functions. Of potential relevance to the etiology of pulmonary hypertension is the recognition that pulmonary capillary endothelial cells can add, remove, activate and inactivate a

number of vasoactive compounds (Fishman and Pietra, 1974; Gillis and Roth, 1976; Said, 1982). These include biogenic amines, prostaglandins, angiotensin and bradykinin. Because MCT causes changes in endothelial cell structure, it might alter function as well.

5-Hydroxytryptamine (5HT) is removed from the pulmonary circulation by a facilitated transport system in the endothelial cell. The transport process is saturable, Na^+-dependent and temperature and ouabain sensitive, suggesting an active carrier-mediated process (Junod, 1972; Iwasawa et al., 1973; Pickett et al., 1975). Evidence for a carrier was also provided by experiments in which uptake of 5HT was reduced by concurrent perfusion with cocaine, phenoxybenzamine, imipramine, or chlorpromazine, drugs known to inhibit 5HT uptake in other systems (Junod, 1972; Iwasawa and Gillis, 1974; Steinberg et al., 1975). Aminophylline, phentolamine and propranolol did not inhibit uptake of 5HT (Iwasawa and Gillis, 1974). Metabolic inhibitors such as cold, anoxia, cyanide, 2-deoxyglucose and iodoacetate all reduced uptake, implying that the process required energy (Junod, 1972; Iwasawa et al., 1973; Steinberg et al., 1975). After uptake into lung cells, 5HT is metabolized by monoamine oxidase (Alabaster and Bakhle, 1970, Junod, 1972; Pickett et al., 1975). Inhibition of monoamine oxidase does not alter initial uptake, suggesting that uptake is the rate-limiting step in removal and inactivation of 5HT by the lung (Alabaster and Bakhle, 1970; Junod, 1972). A similar but separate carrier-mediated process has been identified for norepinephrine in lung (Iwasawa and Gillis, 1974; Nicholas et al., 1974). The endothelial cell is the major site of 5HT uptake in the lung, based on autoradiographic (Strum and Junod, 1972; Cross et al., 1974)

and fluorescent histochemical evidence (Iwasawa et al., 1973). Further, experiments in the dog have suggested that in the lung, the capillary bed is the major site of 5HT removal (Rickaby et al., 1980). Cultured endothelial cells from bovine pulmonary artery demonstrate carrier-mediated uptake of 5HT similar to intact lungs (Block and Stalcup, 1981). 5HT uptake is not a function unique to pulmonary endothelial cells, however. Isolated endothelial cells from epididymal fat pads (Robinson-White et al., 1981) and thoracic aorta (Junod and Ody, 1977) also have been shown to take up 5HT with the characteristics described above.

The physiological function of 5HT clearance by the lung is not known. Vane speculated that the lung, fixed between the heart and the systemic circulation and receiving the entire cardiac output, might protect the arterial circulation from inappropriate vasoactivity or thrombosis by removing locally acting agents and allowing circulating hormones to pass unaltered (Vane, 1969). Perturbation of the clearance process could alter the concentration of 5HT in the pulmonary venous and systemic arterial circulations. A change in 5HT concentration has a number of potential consequences. 5HT is a vasoconstrictor in many species, including the rat, dog, and rabbit (Rickaby et al., 1980; DeClerck and Van Nueten, 1982; Van Nueten et al., 1982). In the pulmonary bed in these species, both arterial and venous vasoconstriction have been observed (Tucker and Rodeghero, 1981; Tucker et al., 1982; Rickaby et al., 1980; Ahmed and Harrison, 1964; Iwasawa et al., 1973). In addition, 5HT can potentiate the response of a vessel to other vasoconstrictors such as angiotensin II

(Van Nueten et al., 1982). 5HT also increases capillary permeability (Rowley, 1964; Majno and Palade, 1961). In many species 5HT causes platelet aggregation and enhances the response of platelets to other aggregating agents (DeClerck and Herman, 1983). In the rat, 5HT does not stimulate aggregation, but it will enhance the response of the platelets to other aggregating agents (Calkins et al., 1974; Doni, 1980).

Several agents which damage pulmonary endothelium have also been shown to decrease 5HT clearance by the lung. These include hyperoxia (Block and Fisher, 1977), superoxide (Cook et al., 1982), alpha-naphthylthiourea (Block and Schoen, 1981), bleomycin (Catravas et al., 1981), and paraquat (Roth et al., 1979). It has been estimated that a very small degree of inhibition of clearance can greatly increase the concentration of 5HT reaching the left atrium (Gillis and Pitt, 1982). Accordingly, a slight inhibition of 5HT uptake may result in significantly increased concentrations of 5HT which, by its actions on the vasculature, may have pathophysiological effects.

MCT, like other compounds which damage endothelial cells, reduces the amount of perfused 5HT removed and metabolized by isolated lungs (Gillis et al., 1978; Huxtable et al., 1978). Monoamine oxidase activity was not altered by MCT treatment. This suggests that the decreased removal was due to alteration in the transport process. MCT did not inhibit 5HT uptake when the two compounds were co-perfused in isolated lungs from control animals. Like the pulmonary hypertension, MCT-induced depression of 5HT removal was not an immediate effect. Seven days after the start of MCT ingestion,

5HT removal by rat lungs was normal. After 14 days or longer, the removal was decreased (Roth et al., 1981).

Norepinephrine is another biogenic amine removed from the pulmonary circulation, apparently by a different carrier than 5HT (Nicholas et al., 1974; Iwasawa and Gillis, 1974). There are differing reports on the effects of MCT on this uptake process. One report indicated a 60% decrease after 3 weeks MCT ingestion (Gillis et al., 1978) while another study using the same treatment regimen saw no decrease in uptake (Huxtable et al., 1978). However, the study showing no effect measured uptake in isolated lungs perfused at room temperature rather than body temperature. Since these uptake processes are temperature dependent (Iwasawa et al., 1973), perfusing at a lower temperature may have blunted responses and obscured any differences.

Angiotensin-converting enzyme (ACE) activity is located on the luminal surface of the pulmonary capillary endothelial cells. ACE transforms the precursor angiotensin I to angiotensin II, a potent vasoconstrictor, and inactivates bradykinin (Ryan and Ryan, 1978). Bradykinin is a pulmonary arterial vasodilator but constricts bronchioles and pulmonary veins. It also increases pulmonary vascular permeability (Pang et al., 1982). ACE activity was decreased in lung homogenates after a single injection of MCT (Kay et al., 1982). In contrast, Huxtable et al. (1978) did not see a decrease in ACE activity in the isolated perfused lung. Whether the difference between these studies was due to the use of lung homogenates vs. the intact organ or the lower temperature used for the isolated lung studies is not clear.

Another biochemical indication of lung injury after MCT treatment was the elevation in lactate dehydrogenase (LDH) activity and protein concentration in bronchopulmonary lavage fluid (Roth et al., 1981). LDH is a cytosolic enzyme and usually only very low activity can be detected free in the airways (Roth, 1981). Elevated activity in the cell-free lavage fluid suggests that alveolar damage has occurred. Along with the rise in LDH activity, the protein concentration was increased (Roth et al., 1981) suggesting that cellular disruption and/or vascular leakage had occurred.

Another biochemical indication of pulmonary changes was the demonstration that collagen synthesis in the pulmonary artery was increased in rats with MCT-induced pulmonary hypertension (Kameji et al., 1980). Collagen synthesis in sections of aorta from MCT-treated rats was not altered, suggesting that the change was specific to the pulmonary vasculature, and thus related to the pulmonary hypertension. In fact, the increased collagen synthesis may reflect ongoing hypertrophy and/or hyperplasia in the pulmonary vessels. Meyrick and Reid (1982) have reported increased collagen in the adventitia of the pulmonary vessels of rats fed Crotalaria spectabilis seeds.

4. Metabolic Activation

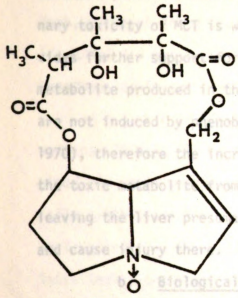
a. Metabolism

Pyrrolizidine alkaloids undergo primary metabolism by hydrolysis, hydroxylation, N-oxidation, and dehydrogenation. Hydrolysis and hydroxylation are considered to be primarily detoxification pathways (Lalich and Ehrhart, 1962; Bull et al., 1968; Jago et al., 1969; McLean, 1970). N-oxidation is perhaps the most significant

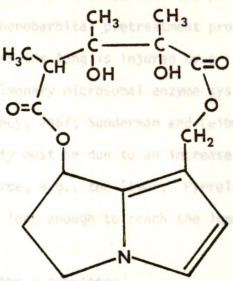
detoxification pathway (Mattocks and White, 1971). The N-oxides of the pyrrolizidines are relatively non-toxic (see below) and readily excreted because of their high water solubility. By contrast, dehydrogenation to pyrrole forms results in toxicity (Mattocks, 1968; Mattocks and White, 1971; Mattocks, 1972).

Monocrotaline N-oxide and the pyrroles, dehydroretronecine (RTP) and dehydromonocrotaline (MCTP) are the major metabolites of MCT which have been studied. Their structures are shown in Figure 2. The formation of the N-oxide and unspecified pyrrole metabolites by rat liver has been shown in vitro (Mattocks, 1968; Mattocks and White, 1971) and in vivo (Mattocks, 1972; Allen et al., 1972). Although the N-oxide can be easily converted chemically to MCTP (Mattocks, 1969), this does not seem to happen in vivo. The ability to transform MCT to N-oxide and pyrroles resides in the microsomal fraction of liver homogenates (Mattocks and White, 1971), and such fractions will not convert N-oxides of various pyrrolizidines to the corresponding pyrroles (Mattocks and White, 1971; Chesney and Allen, 1973b). Lung tissue does not produce detectable pyrroles after incubation of either microsomes (Mattocks and White, 1971) or lung slices (Mattocks, 1968; Armstrong and Zuckerman, 1970) with pyrrolizidine alkaloids.

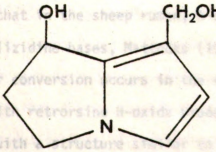
Metabolic activation of monocrotaline is required for toxicity to occur. Inhibition of the mixed function oxidase enzyme system in the liver by treating with chloramphenicol greatly reduces the pyrrole levels in blood, liver, heart and lung of MCT-treated rats and protects the animals from lung and liver toxicity (Allen et al., 1972). If liver metabolic enzymes are induced by phenobarbital



N-OXIDE



DEHYDROMONOCROTALINE



DEHYDRORETRONECINE

Figure 2. Structure of major microsomal metabolites of monocrotaline.

pretreatment, the toxicity of MCT is increased (Mattocks, 1972; Allen et al., 1972) and pyrrole production by liver microsomes is greatly enhanced (Mattocks and White, 1971). The observation that the pulmonary toxicity of MCT is worsened by phenobarbital pretreatment provides further support for the idea that the lung is injured by a metabolite produced in the liver: Pulmonary microsomal enzyme systems are not induced by phenobarbital (Conney, 1967; Sunderman and Leibman, 1970), therefore the increased toxicity must be due to an increase in the toxic metabolite from another source, e.g., the liver. Pyrrole leaving the liver presumably survives long enough to reach the lung and cause injury there.

b. Biological effects of the metabolites:

1. MCT N-oxide. Early reports suggested that the N-oxides of MCT (Schoental and Magee, 1959) and fulvine (Barnes et al., 1964) were just as damaging to the lung and liver as the parent compounds. In both cases, the N-oxide was administered by oral gavage. Based on a report that in the sheep rumen, N-oxides were converted to the original pyrrolizidine bases, Mattocks (1971) tested the possibility that a similar conversion occurs in the rat gut. Incubation of rat gut contents with retrorsine N-oxide produced retrorsine, a pyrrolizidine alkaloid with a structure similar to MCT. Furthermore, when liver pyrrole concentrations were measured after oral administration of N-oxide, the same maximal concentration as resulted from the parent compound was achieved. After intraperitoneal administration of N-oxide, the maximum liver pyrrole concentration was only one-fourth of that seen after the oral dose. This suggests that oral administration of the N-oxides is not appropriate for determining the effects

of the actual N-oxides since the body is likely responding to the parent compound instead. Biological effects of intravenous administration have not been reported. Unpublished studies (Bruner and Roth, personal communication) indicated that a low dose of MCT N-oxide (5 mg/kg) did not cause a decrease in body weight gain or pulmonary hypertension in the treated rats.

2. Dehydroretronecine. According to Hsu and coworkers (1973a), RTP is the major pyrrolic metabolite in the liver, blood and urine after a single large dose of MCT. Injection of chemically synthesized RTP in very large doses (70-90 mg/kg, s.c.) caused no lung injury. The pathology that was observed consisted of gastrointestinal ulceration and hemorrhage and diffuse megalocytosis and focal subcapsular scarring in the liver.

In contrast to this work, right ventricular hypertrophy, increased lung weight and loss of body weight have been demonstrated after 3 weeks of daily subcutaneous injections of 4 mg RTP/kg (Huxtable et al., 1978). This treatment regimen also decreased 5'-nucleotidase activity (associated with endothelial cell membranes). This effect was not seen by the same investigators after MCT administration, suggesting that RTP was not the mediator of MCT pulmonary toxicity. The observation that RTP can cause pulmonary hypertension suggests, however, that it may contribute to MCT-induced pulmonary hypertension.

3. Dehydromonocrotaline. MCTP is presumed to be the major toxic metabolite of MCT because injection of the chemically synthesized compound mimics the effects of MCT. Injection of the

pyrrole form of the closely related alkaloid retrorsine into a superior mesenteric vein results in hepatic damage while injection of MCTP into the tail vein results in pulmonary hypertension without any hepatic damage (Chesney et al., 1974). Other morphological changes characteristic of MCT damage have also been observed after MCTP administration: thickened alveolar walls, increases in macrophages and mast cells, platelet and fibrin thrombi in blood vessels, endothelial cell swelling, alveolar edema and hemorrhage, medial thickening of pulmonary blood vessel walls and right ventricular hypertrophy (Butler et al., 1970; Butler, 1970; Chesney et al., 1974; Lalich et al., 1977).

5. Pharmacokinetics

Little is known about the pharmacokinetics of MCT. Pyrrole concentrations reach a maximum in tissues 30-60 minutes after oral or parenteral administration of MCT (Mattocks, 1972; Allen et al., 1972), then drop rapidly to about half the maximal level. The pyrrole concentration drops slowly and gradually from this point to low but detectable levels at 48 hours. Pyrrole levels at longer intervals after injection have not been reported. Most of the MCT dose was excreted in the urine by 24 hr after administration (Hayashi, 1966; Mattocks, 1968). Using ^{3}H-labelled MCT, Hayashi (1966) reported finding 50-70% of the administered dose in the urine (with intact pyrrolizidine ring) and 30% of the label in the bile (not associated with an intact pyrrolizidine ring structure). This investigator also found ^{3}H-labelling in the liver 72 hours after dosing. Other organs (unspecified by the author) had no significant radioactivity at this time.

B. Human Pulmonary Hypertension

1. General

The basic definition of pulmonary hypertension (PH) is an increase in pulmonary arterial pressure. Chronic pulmonary hypertension is usually the result of some underlying disease. Congenital heart diseases which cause a systemic to pulmonary shunt are a common cause, particularly in children. Chronic hypoxia (e.g., high altitude) is another relatively frequent cause. Other disease entities are much less common but still well characterized. Hepatic portal cirrhosis is often accompanied by pulmonary hypertension as is mitral valve disease. Pulmonary thromboemboli and pulmonary veno-occlusive disease can also result in pulmonary hypertension (Wagenvoordt and Wagenvoordt, 1977). When all of these causes have been ruled out, a finite number of cases of confirmed pulmonary hypertension still remain. These are designated as primary pulmonary hypertension (PPH). It is unlikely that there is a single underlying cause or mechanism which would explain all cases of PPH but there may be two or three major pathways involved.

The criteria for a diagnosis of PPH are (1) an increase in pulmonary arterial pressure, (2) normal pulmonary wedge pressure, (3) lack of known causes, and (4) characteristic morphological changes in biopsy/autopsy samples (Voelkel and Reeves, 1979).

Primary pulmonary hypertension is not a common disease but difficulty in diagnosis and lack of effective therapy make it a serious problem. There are some 1000 case reports in the literature (Voelkel and Reeves, 1979; Wagenvoordt and Wagenvoordt, 1977).

Reliable estimates of the incidence of PPH are not available, however it is suspected to be more common than the documented cases would indicate, owing to the difficulty of confirming the diagnosis (Voelkel and Reeves, 1979). Overall, there is a greater incidence of PPH in females than males (Wagenvoordt and Wagenvoordt, 1970). Prior to adolescence, there is no difference in the number of cases between the sexes (Voelkel and Reeves, 1979). A number of theories have been propounded to explain the greater susceptibility of women but none of them satisfactorily fit all of the data.

The symptoms of PPH are quite nonspecific, especially in the early stages. Dyspnea, chest pain, weakness, fatigue and syncope upon exertion are the most common complaints. The disease usually progresses to right ventricular failure and results in death (Ross, 1980; Fein and Frishman, 1980). Diagnosis of PPH is difficult and usually only achieved in the later stages of the disease. Roentgenography may reveal dilatation of large pulmonary vessels and enlargement of the right ventricle. Much of the diagnosis relies on eliminating other possible causes of PH, such as mitral valve disease or congenital heart defects. The only way to confirm the diagnosis is by pulmonary angiography and cardiac catheterization. There is a high mortality rate as a result of the cardiac catheterization procedure in patients with PPH (Voelkel and Reeves, 1979) so this is not done routinely.

A wide variety of therapeutic approaches have been used in PPH, without overwhelming success. Because the etiology is unknown, the approach to therapy has been largely empirical, usually aimed at reducing the pulmonary vascular resistance and providing supportive

treatment. Fein and Frishman (1980) have recently reviewed this subject. Successful intervention was usually achieved only in the early stages of the disease. Pharmacological approaches which have been successful include cholinergic and beta-adrenergic stimulation, alpha-adrenergic blockade, vasodilators, and prostaglandin biosynthesis inhibitors. Antihistamines, reserpine, corticosteroids and calcium channel blockers have not shown much promise. It is unlikely that all cases of PPH have the same cause, which may explain the lack of uniform success of any therapeutic approach. It is possible, however, that there are common mechanisms underlying a number of cases of PPH. An understanding of even a part of the etiology of the disease would help in the design of more effective therapy.

2. Pathology

The pathological changes in the lung in PPH have been described in detail in three large studies of autopsy material (Wagenvoordt and Wagenvoordt, 1970; Walcott et al., 1970; Watanabe and Ogata, 1976). Medial hypertrophy and muscularization of non-muscular vessels occurred uniformly. A striking change, occurring in over 70% of the patients studied (Wagenvoordt and Wagenvoordt, 1970), is the plexiform lesion. This is seen in histological section as a local dilatation of a small muscular pulmonary artery containing a plexus of small slits separated by strands of intimal cells. Affected vessels frequently showed signs of inflammatory cell infiltration and remnants of necrotizing arteritis. Another frequent lesion was intimal proliferation, which often progresses to concentric laminar intimal fibrosis. Arteritis, necrotizing arteritis, and fibrinoid necrosis

have also been reported. Thrombotic lesions and thromboemboli were also seen but not commonly enough in any one lung to suggest a thromboembolic origin for the PH. At the ultrastructural level, endothelial cell swelling, obstruction of capillaries and thickened capillary basement membranes were observed. Decreased filling of small peripheral arterioles by radioopaque media has been demonstrated (Anderson et al., 1973) suggesting obstruction or narrowing of the lumen in these vessels.

Along with the morphological changes, an interesting observation on the ability of lungs of patients with increased pulmonary vascular resistance to remove biogenic amines from the circulation has been described. During cardiac catheterization procedures, extraction of norepinephrine was not detectable across the pulmonary bed in 7 patients with increased pulmonary vascular resistance. By comparison, patients with normal pulmonary vascular resistance extracted about 25% of the blood norepinephrine in a single pass through the vasculature (Sole et al., 1979). A similar observation was made in children with PH (Gewitz et al., 1982). In contrast, increased removal of NE has been reported in patients with elevated PAP, but in this study PVR was not measured (Gillis et al., 1974). An indication that the decreased ability of the lung to remove biogenic amines might have physiological consequences is provided by the measurement of plasma norepinephrine levels (Kondo et al., 1981). Plasma concentrations of norepinephrine were almost doubled in patients with confirmed pulmonary hypertension compared to patients with other cardiovascular diseases.

3. MCT as a Model for Human PPH

The similarities between MCT-induced PH in rats and human PPH are striking. Both have the major effect of a progressive increase in pulmonary arterial pressure, accompanied by right ventricular hypertrophy. The pathology commonly includes medial thickening of vessels and extension of smooth muscle into normally non-muscular pulmonary arterioles. Both also include vascular arteritis, intimal proliferation and fibrosis, thromboembolization, endothelial cell swelling and capillary obstruction and decreased luminal size in small pulmonary vessels. Additionally, lungs from MCT-treated rats and patients with PH have a decreased ability to remove biogenic amines from the circulation.

One potential discrepancy between the animal model and the human disease lies in the apparent lack of plexiform lesions in the former. These lesions are very characteristic of human PPH but have been described only once in the MCT model (Watanabe and Ogata, 1976). This may not be a serious criticism. First, it is possible that plexiform lesions existed in MCT-treated lungs but were not usually recognized as such. Most of the detailed histopathology on MCT toxicity was published before the descriptions of the plexiform lesion as a significant lesion in the human disease. Secondly, the plexiform lesions are believed to be slow to develop and to be a consequence of necrotizing arteritis (Wagenvoordt and Wagenvoordt, 1977). Necrotizing arteritis has been reported in MCT-treated lungs. However, the duration of experimental studies with MCT has been relatively short, so it is possible that there simply has not been time for plexiform

lesions to develop. Overall, the similarities in the pathological effects correspond so well that MCT seems to be a reasonable model. Elucidation of the etiology of the PH after MCT administration may provide information which can be applied to diagnosis and treatment of the human disease.

C. Potential Mechanisms of MCT-Induced Pulmonary Hypertension

The mechanism(s) by which MCT leads to pulmonary hypertension are not known. The major toxic properties of MCT are probably due to its reactive hepatic metabolite, MCTP (see above). It seems likely that the initial interaction between MCTP and the lung is one of covalent binding of the pyrrole to the tissue. Mattocks (1968) has proposed a mechanism by which pyrrole moieties can bind to nucleophilic compounds. Covalent interactions between RTP and tissue macromolecules have been demonstrated in vitro and in vivo (Hsu et al., 1975; Robertson, 1982). Binding of MCTP to DNA and other nucleophiles has been shown in vitro (Mattocks, 1969; White and Mattocks, 1972), but has not been reported in vivo.

The events linking covalent binding and pulmonary hypertension, if a connection even exists, are unknown at this time. Several possible origins for the PH have been proposed. One theory, based on the apparent venous constriction after MCT administration (Smith and Heath, 1978) suggests that the pyrrole caused a direct vasoconstriction which resulted in increased pulmonary vascular resistance and could have caused PH. A problem with this theory is that these studies were done in rats fed Crotalaria seeds, thus receiving a

continuous supply of MCT. Pulmonary hypertension also results from a single injection of either MCT or MCTP and is not detected until long after the last vestiges of MCT or MCTP are eliminated from the blood (e.g., Ghodsi and Will, 1981). It is unlikely that a transient vasoconstriction while the alkaloid was actually in the body would be sufficient to cause the observed chronic progressive changes.

Another theory for the origin of MCT-induced PH is that it is caused by hypoxemia (Turner and Lalich, 1965; Kay et al., 1969; Chesney et al., 1974). This is based on the premise that the thickened alveolar walls would decrease gas diffusion. Acute alveolar hypoxia causes pronounced pulmonary vasoconstriction (Berkov, 1974; McMurtry et al., 1976) and chronic hypoxia can lead to pulmonary hypertension (Wagenvoordt and Wagenvoordt, 1977; Rabinovitch et al., 1979). However, rats fed Crotalaria seeds do not exhibit hypoxemia until immediately prior to death, long after the pulmonary hypertension has been established (Meyrick et al., 1980). Local ventilation-perfusion inequalities may result in some regions of hypoxia in the lung but hypoxic vasoconstriction is probably not the major cause of MCT-induced PH.

A potential role for 5HT in the etiology of MCT-induced PH has also been proposed, based on circumstantial evidence (Turner and Lalich, 1965; Kay et al., 1976). Mast cells reportedly accumulate in the lungs of MCT-treated rats (Takeota et al., 1962; Valdivia et al., 1967). The recognition that rat mast cells contain large amounts of 5HT (Benditt et al., 1955) and that 5HT is a potent pulmonary vasoconstrictor (Rickaby et al., 1980; Tucker and Rodeghero, 1981) has led

to the suggestion that 5HT-induced vasoconstriction results in PH. The observation that removal of 5HT is decreased in perfused lungs from MCT-treated rats has also led to the speculation that 5HT may be important in the pathogenesis of MCT-induced PH (Gillis et al., 1978; Huxtable et al., 1978), since decreased inactivation would favor elevated intravascular 5HT concentrations. Chronic administration of 5HT to rabbits has produced pulmonary hypertension with morphological changes similar to those seen in MCT-treated rats (Ahmed and Harrison, 1964). Studies by Kay and Heath (1969) were unable to confirm the reported increase in mast cell number or to demonstrate changes in platelet and plasma 5HT concentrations. These studies do not rule out a role for 5HT but suggest that it may not be the only cause of MCT-induced PH.

As discussed previously, it is well established that MCT must be converted to MCTP in order to cause lung injury, and it is likely that MCTP is produced by the liver but not the lung. It is possible that, instead of or in addition to direct damage of endothelial cells in the lung, MCTP released from the liver damages an element or elements in the blood. When blood cells leave the liver, the next capillary bed to which they are exposed is that of the lung, making lung injury a reasonable sequel to MCTP-induced damage of blood cells. The platelet was chosen for the thrust of this investigation for several reasons. First, platelet-containing thrombi were a common finding after MCT or Crotalaria seed intoxication. Second, the platelet, perhaps more than other types of blood cells, might injure lung tissue by a number of potential mechanisms. Finally, preliminary experiments indicated that

a single injection of MCT greatly decreased the circulating platelet count in treated rats (see "Results" section; Hilliker et al., 1982), suggesting that MCT had some effect on platelets.

The purpose of this project was to test the hypothesis that platelets play a role in the development of MCT-induced pulmonary hypertension and to characterize that role. Figure 3 depicts schematically a variety of mechanisms by which platelets may be involved in the pathogenesis of MCT-induced pulmonary hypertension.

MCTP could react with the platelet membrane directly, causing aggregation and formation of microemboli which could lodge in the lung and increase the pulmonary arterial pressure (PAP) by occluding small vessels. It has been demonstrated in dogs that platelets exacerbate the increase in PAP caused by microembolization with glass beads (Mlczoch et al., 1977). In addition, thrombocytopenia ameliorates the increase in PAP seen after injection of a large embolus into the right atrium (Cade, 1975). However, in these cases the increase in PAP is transient, suggesting that microembolization alone would not lead to right ventricular hypertrophy, which requires a sustained increase in PAP to develop. Accordingly, if platelets act through formation of emboli, a continuous insult with microemboli must occur or the emboli must lead to lasting vessel wall damage in order to result in chronic PH and right ventricular hypertrophy. A continuous barrage of emboli seems unlikely because MCT and its metabolites are largely eliminated from the body by 48 hours after injection (Hayashi, 1966; Mattocks, 1972) while increases in PAP are not detectable until 7-14 days after injection (Ghodsi and Will, 1981; Kay et al., 1982). The emboli might

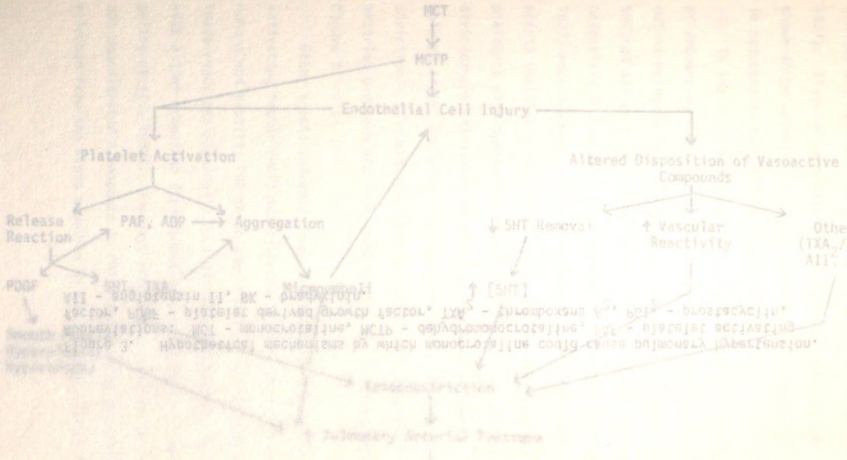

MCT
MCTP
Endothelial Cell Injury
Platelet Activation
Altered Disposition of Vasoactive Compounds
Release Reaction
PAF, ADP
Aggregation
↓ 5HT Removal
↑ Vascular Reactivity
Others (TXA2/PGI2 AII, BK)
PDGF
↑ [5HT]
Vasoconstriction
↑ Pulmonary Arterial Pressure
Right Ventricular Hypertrophy
Right Heart Failure

Figure 3.

Figure 3. Hypothetical mechanisms by which monocrotaline could cause pulmonary hypertension. Abbreviations: MCT - monocrotaline, MCTP - dehydromonocrotaline, PAF - platelet activating factor, PDGF - platelet derived growth factor, TXA_2 - thromboxane A_2, PGI_2 - prostacyclin, AII - angiotensin II, BK - bradykinin.

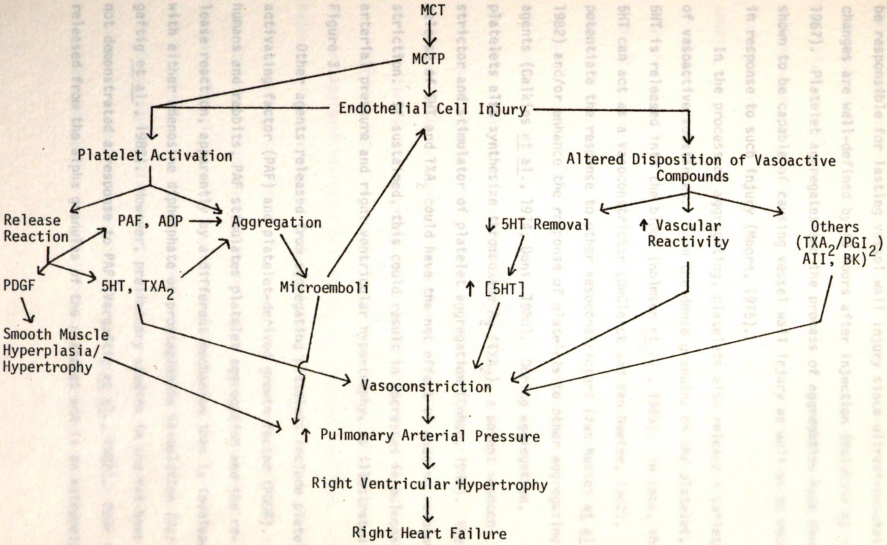

MCT
MCTP
Endothelial Cell Injury
Platelet Activation
Altered Disposition of Vasoactive Compounds
Release Reaction
PAF, ADP
Aggregation
↓ 5HT Removal
↑ Vascular Reactivity
Others (TXA_2/PGI_2) AII, BK)
PDGF
5HT, TXA_2
Microemboli
↑ [5HT]
Smooth Muscle Hyperplasia/ Hypertrophy
Vasoconstriction
↑ Pulmonary Arterial Pressure
Right Ventricular Hypertrophy
Right Heart Failure

Figure 3.

be responsible for lasting vessel wall injury since ultrastructural changes are well-defined by 48 hours after injection (Valdivia et al., 1967). Platelet aggregates and the process of aggregation have been shown to be capable of causing vessel wall injury as well as to occur in response to such injury (Moore, 1975).

In the process of aggregating, platelets also release a variety of vasoactive substances. From the dense granules of the platelet, 5HT is released into the blood (Holmsen et al., 1969). In rats, the 5HT can act as a vasoconstrictor (DeClerck and Van Nueten, 1982), potentiate the response to other vasoconstrictors (Van Nueten et al., 1982) and/or enhance the response of platelets to other aggregating agents (Calkins et al., 1974; Doni, 1980). During aggregation, platelets also synthesize thromboxane A_2 (TXA_2), a potent vasoconstrictor and stimulator of platelet aggregation (Cohen, 1980). The release of 5HT and TXA_2 could have the net effect of causing vasoconstriction. If sustained, this could result in increases in pulmonary arterial pressure and right ventricular hypertrophy, as illustrated in Figure 3.

Other agents released from aggregating platelets include platelet-activating factor (PAF) and platelet-derived growth factor (PDGF). In humans and rabbits, PAF stimulates platelet aggregation and the release reaction, apparently by a different mechanism than is involved with either adenosine diphosphate or prostaglandin stimulation (Vargaftig et al., 1982). However, preliminary studies in the rat have not demonstrated a response to PAF (Vargaftig et al., 1982). PDGF is released from the alpha granules of the platelet and is an extremely

potent stimulator of smooth muscle cell proliferation *in vitro* (Ross and Vogel, 1978). Its function *in vivo* is not yet known. It is possible that PDGF is responsible for all or part of the smooth muscle cell hypertrophy/hyperplasia observed after MCT administration and thus contributes to the structural component of the pulmonary hypertension.

It is reasonable to expect that damage to endothelial cells will alter their action on vasoactive compounds. It has already been shown that MCT decreases the ability of pulmonary endothelial cells to remove 5HT from the circulation (Gillis *et al.*, 1978; Roth *et al.*, 1981). Decreased removal may result in increased concentrations of 5HT in the blood. This may promote vasoconstriction, platelet aggregation or enhance the sensitivity of the vascular smooth muscle to other vasoconstrictors (Van Nueten *et al.*, 1982), all of which could ultimately cause vasoconstriction and hence, an increase in PAP.

The disposition of vasoactive compounds other than 5HT may also be affected by MCT. The endothelium produces the vasodilator and platelet aggregation inhibitor, prostacyclin (Moncada and Vane, 1979). Decreases in its synthesis could alter the relative concentrations of vasoconstrictors and vasodilators present in the blood leading to a net increase in vasoconstrictors and, thus, vasoconstriction. Endothelial cells also convert angiotensin I to angiotensin II and inactivate bradykinin (Ryan and Ryan, 1978). A decrease in angiotensin converting enzyme activity might mean a decrease in AII production which would reduce the amount of vasoconstrictor in the blood, but a concomitant increase in bradykinin would accompany it. Bradykinin

causes venous constriction and capillary edema (Rowley, 1964), which could contribute to the pathological process.

Another possible consequence of endothelial cell injury is that reactivity to vasoactive compounds may be altered in affected lungs. An increase in reactivity could cause a greater vasoconstriction to a given stimulus or reflect a lower threshold for vasoconstrictors. An increase in capillary permeability as a result of endothelial cell injury might cause these results by increasing the concentration of vasoconstrictors at the smooth muscle cell. One of the earliest changes observed after MCT administration is an increase in capillary permeability to Thorotrast^{R} (Valdivia et al., 1967).

It is unlikely that MCT induces pulmonary hypertension by perturbing homeostasis of a single vasoactive system in the lung. It is more probable that the disease arises from a complex of interactions of a variety of metabolic and functional systems. The purpose of this project was to explore the possibility that platelets and/or 5HT might be involved in the etiology. These two entities were chosen for investigation because of their potential for extensive interaction with one another and the pulmonary vasculature.

D. Specific Aims

1. To develop a treatment regimen which leads to pulmonary hypertension within 2-3 weeks after a single injection of MCT and to characterize the development of the effects of MCT on the lung.

2. To examine the possibility that MCT metabolites damage pulmonary endothelial cells directly.

3. To determine whether platelets are necessary for the development of MCT-induced pulmonary hypertension by examining the effects of MCTP in thrombocytopenic animals.

4. To examine how platelets are involved in MCTP-induced PH by determining platelet aggregation responses in platelet-rich plasma from MCTP-treated rats, observing the effects of platelet-inhibiting drugs on MCTP-induced PH and evaluating the effects of MCTP on fawn-hooded rats, a strain with a specific platelet defect.

5. To determine whether the reactivity of the pulmonary vascular bed is altered by MCTP-induced PH.

MATERIALS AND METHODS

A. Animals

Male, Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) and male CD/COBS rats (Sprague-Dawley derived, Charles River Laboratories, Portage, MI) were used in these studies unless otherwise noted. Male, fawn-hooded rats were obtained from the colony maintained by Dr. W. Jean Dodds at the New York Department of Public Health laboratories (Albany, NY). Rats for all *in vivo* treatments with MCT or MCTP were 225-275 grams at the time of treatment. For the platelet aggregation studies slightly larger rats (275-325 g) were treated. All rats were housed on corn cob bedding in plastic cages in the Laboratory Animal Care Service (LACS) facilities in the Life Sciences and Clinical Center Buildings. When possible, animals were kept in an animal isolator (Contamination Control, Inc., Lansdale, PA) which provided HEPA-filtered air.

All rats were kept in temperature-controlled rooms with an alternating 12 hour light/dark cycle. Food (Wayne Lab Blox^{R}, Continental Grain Company, Chicago, IL) and tap water were available *ad libitum*.

An adult female Nubian goat was used for the preparation of anti-rat platelet antibody. The goat was kept in a box stall in the LACS goat barn. Loose hay and water were freely available and alfalfa pellets were provided once a day.

B. Monocrotaline Treatment

All experiments were designed so that control and treated animals were evaluated on the same day.

1. Drinking water

Monocrotaline was dissolved in a minimal volume of 0.2 M HCl. The pH was adjusted to 7 using 2 M NaOH then the solution was adjusted with double distilled water to a final concentration of 22 mg MCT/ml. This stock solution was diluted 1000-fold with tap water to make drinking water for the rats containing 22 μg MCT/ml. Controls drank tap water.

2. Single injection

MCT was dissolved in 0.2 M HCl and the pH was adjusted to 7 using 2 M NaOH. The solution was brought to volume with double distilled water to make a final concentration of 60 mg/ml. This solution was administered subcutaneously. Control rats received an equivalent volume of 0.9% saline.

3. Dehydromonocrotaline

MCTP was dissolved in N,N-dimethylformamide (DMF) to make a solution containing 10 mg MCTP/ml. Solutions were prepared immediately prior to injection. Rats were treated with 5 mg MCTP/kg body weight or 0.5 ml DMF/kg via the tail vein. Injection of the MCTP or DMF solution was followed by 0.3 ml saline to flush the dose into the vasculature.

C. Synthesis of Monocrotaline Pyrrole (Dehydromonocrotaline)

1. N-oxide preparation

Monocrotaline was converted to its N-oxide by oxidation with hydrogen peroxide (Leisegang and Warren, 1969). MCT (2.0 g) was dissolved in 40 ml methanol and refluxed with 1.7 ml 30% H_2O_2 for 5-7 hours. The refluxed mixture was then stirred at room temperature overnight with 50 mg Adam's catalyst (Platinum (IV) oxide; Mattocks, 1969) to scavenge any excess H_2O_2. The mixture was filtered to remove the Adam's catalyst and the methanol was removed using a Büchi Rotavapor R110 (Brinkmann Instruments, Westbury, NY). Approximately 20-30 ml acetone dried over sodium sulfate, was added to the resultant yellow oil until white crystals began to form. The mixture was refrigerated at least 24 hours and the crude product was isolated by vacuum filtration. The N-oxide was recrystallized from absolute ethanol. Typical yields of recrystallized material were 69%. The N-oxide was stored at room temperature in a dessicator.

2. MCTP preparation

MCTP was prepared as described by Mattocks (1969) by the reduction of the N-oxide using ferrous sulfate in a hot, concentrated solution in methanol. Sodium fluoride was added to accelerate the reaction and give cleaner products. Addition of excess ether minimized the reaction of the pyrrole product(s) with the methanol as well as precipitating the inorganic material. The ether filtrate was concentrated by evaporation under N_2 to allow recovery of the product. The specifics of the synthesis procedure follow.

All glassware used had the inside surface exposed to fumes from concentrated NH_4OH. The glassware was then heated 10 min in a steam heated oven and cooled in a dessicator. Anhydrous ethyl ether was further dried by passing through a 5 cm neutral alumina (Brockmann Activity I) column (Dasler and Bauer, 1946). The ether was then bubbled with dry N_2 prior to use. To prepare ultradry methanol, 1.7 g of warm, dried Mg turnings were added to 0.07 g I_2 in 25 ml methanol in a small flask. This mixture was heated and stirred until the brown I_2 color disappeared. The mixture was transferred to a 500 ml round bottom flask and refluxed with 350 ml methanol for 3 hours. The ultra-dry methanol was distilled, discarding the first 30 ml of distillate, and collected over molecular sieves to remove any trace amounts of water. Ultra-dry methanol was also run through an alumina column (0.5 x 2 cm, neutral alumina, Brockmann Activity I, immediately prior to use).

To prepare the MCTP, 250 mg of recrystallized N-oxide was dissolved in 1 ml of ultra-dry MeOH. In a separate flask, 500 mg freshly powdered $FeSO_4 \cdot 7H_2O$ and 75 mg NaF were slurried in 1 ml ultra-dry MeOH. The two flasks were swirled on a hot plate until the N-oxide solution was hot and the $FeSO_4$ solution was boiling. The $FeSO_4$/NaF slurry was then poured into the N-oxide and swirled for 5-10 seconds. Approximately 200 ml of the dry ether was added in 3 or 4 additions while continuing to swirl the mixture. The ether was separated from the inorganic precipitate by gravity filtration through fluted filter paper. A light stream of dry N_2 was directed over the filtrate during filtration. The volume of the filtrate was reduced to

about 10 ml by evaporation at room temperature under dry N_2. The concentrated filtrate plus 10 ml of dry ether used to wash the evaporating vessel was eluted through a 0.5 x 2 cm column of neutral alumina (Activity I). The eluate was evaporated to about 1 ml under N_2 and stored tightly covered at -20°C for 12-24 hours to promote crystallization. Crystals were recovered by evaporation of the remaining ether to near dryness, then rapidly transferred to storage vials with a teflon-coated spatula. The vials were flushed with dry N_2, capped, wrapped in aluminum foil and stored at -20°C. Usual yields were 30-60 mg, 13-25% of the theoretical maximum.

3. Confirmation of MCTP structure

The structure of the product was confirmed by direct probe electron impact mass spectrometry. A Finnegan 3200 Gas Chromatograph/Mass Spectrometer was used with an accelerating voltage of 70 eV and temperature programming from 50-300°C. These analyses were kindly done by John Snyder and Dr. W.E. Braselton of this department. The mass spectra were compared to those previously reported for MCTP (Mattocks, 1969; Culvenor et al., 1970).

4. Determination of product purity

Thin-layer chromatography was used for assessment of purity. Whatman LHP-K thin layer plates were run in a solvent system of diethyl ether : methanol : diethylamine (96.8 : 2 : 1.2). Diethyl ether was dried by running through a neutral alumina column (Brockmann Activity I, 1.5 x 4 cm). Ultradry methanol was also run through an alumina column to dry it completely. The diethylamine was stored over NaOH pellets to remove excess water. Samples were prepared in dry

diethyl ether. After development in the solvent, spots were detected by spraying with Ehrlich's reagent and heating. Some plates were sprayed with a 1:1 mixture of methanol and sulfuric acid, then heated, but this was less sensitive than the Ehrlich's reagent so it was not used routinely.

D. Pyrrole Generating System

Rats were treated with 80 mg phenobarbital/kg, i.p., daily for 4 days to induce the mixed function oxidase enzyme systems in the liver (Allen et al., 1972). Under ether anesthesia, the liver was cleared of blood by perfusion with ice-cold 0.9% saline through the hepatic portal vein. Free-hand liver slices (approximately 1 mm thick) were incubated in Krebs-Ringer bicarbonate (KRB) buffer with 930 μg MCT/ml (2.9 mM) and lung slices for 5 hours at 37°C under 95% O_2/5% CO_2. After 5 hours the lung slices were rinsed and transferred to fresh KRB for measurement of 5HT uptake.

E. Ehrlich Assay

A modified Ehrlich reaction (Mattocks and White, 1970) was used to estimate the pyrrole present in tissue slices and incubation medium for the slice studies. Chemically synthesized MCTP was used to construct a standard curve allowing the conversion of absorbance units to quantities of pyrrole. The Ehrlich reagent reacts specifically with pyrrole moieties but it also reacts with a wide variety of pyrrole-containing compounds other than those derived from pyrrolizidines. Thus, while Ehrlich reactivity indicates the presence of pyrroles it

provides no information about the identity or quantity of a specific pyrrole. As a result, concentrations are reported as μg pyrrole/g tissue or μg pyrrole/ml medium, an estimate of the total pyrrole moieties present.

The Ehrlich reagent consisted of 3 g p-dimethylaminobenzaldehyde dissolved in 14% boron trifluoride in methanol, diluted to 100 ml with absolute ethanol. Tissue samples to be assayed were weighed, then homogenized (Polytron, Brinkmann Instruments, Westbury, NY) in 5% mercuric chloride in ethanol, forming insoluble complexes between the pyrrole derivatives and $HgCl_2$. The solids were separated by centrifugation and washed with absolute ethanol. After centrifugation, the pellet was resuspended in 2 ml ethanol and mixed with 2 ml Ehrlich reagent. The tubes were heated 1 minute in a boiling water bath, cooled to room temperature and diluted to 5 ml with ethanol. The solids were pelleted by centrifugation, and the absorbance of the supernatant at 565 and 625 nm was measured in a Beckman DU spectrophotometer with a Gilford Model 252 photometer (Gilford Instrument Laboratories, Oberlin, OH). Tissue blanks were run to correct for endogenous pyrroles. The λ_{max} of the Ehrlich pyrrole complex is 565 nm. The λ_{max} of Ehrlich reagent-treated blood is 625 nm. Absorbance values were corrected for the presence of residual blood in the tissues with the formula described by Mattocks and White (1970):

$$\text{Corrected Abs} = 1.1\ (Abs_{565} - Abs_{625})$$

To measure pyrrole in incubation media or other tissue-free samples, one ml of incubation medium was mixed with one ml of 5%

ascorbic acid in 80% ethanol. One ml Ehrlich reagent was added and the mixture was heated in a boiling waterbath for 1 minute. After cooling to room temperature, 0.1 ml 35% $FeCl_3$ in water was added to inhibit fading of the color of the Ehrlich-pyrrole complex. Ethanol was added to bring the volume of each sample to 5 ml. The absorbance was measured at 565 nm.

F. Uptake Measurements in Lung Slices

1. Preparation of lung slices

Rats weighing 200-300 g were anesthetized with 50 mg pentobarbital/kg, i.p. Heparin (500 U) was injected into the abdominal vena cava. The thoracic vasculature was cleared by perfusing with ice-cold 0.9% saline through the hepatic portal vein. Lung slices 1 mm thick were made with a McIlwain tissue chopper and placed in cold KRB at pH 7.4. Only slices with two cut surfaces were used for assays. The incubation system consisted of 4-5 lung slices (50-100 mg tissue) in 3 ml KRB.

2. 5HT and paraquat uptake into lung slices

For 5HT uptake studies the incubation medium contained 500 μM iproniazid to inhibit monoamine oxidase activity. After 20 minutes preincubation at 37°C under 95% O_2/5% CO_2, either 1 μM [^{14}C]-5HT creatinine sulfate or 10 μM [^{14}C]-paraquat (PQ) was added. 5HT uptake was determined after 5 minutes of incubation, PQ uptake was evaluated after 10 minutes. Slices were rinsed, blotted and weighed, then solubilized in 1.0 ml Soluene 350^R in glass scintillation vials by shaking overnight. Radioactivity in the samples was measured in a

Beckman LSC-100 liquid scintillation counter in 10 ml 3a20^{R} scintillant. Counting efficiency was determined using the external standard channels ratio method. Uptake results were expressed as pmol amine/mg tissue·minute.

G. Isolated Perfused Lung (IPL)

1. Surgery

Rats were anesthetized with 50 mg/kg sodium pentobarbital, i.p. The trachea was cannulated with PE-240 tubing (Clay Adams, Passipanny, NJ). The abdomen was opened and 500 U heparin was injected into the inferior vena cava. After one minute to allow the heparin to distribute, the diaphragm was cut along the abdominal wall and the rib cage was cut open. The thymus was teased out and the pulmonary artery was cannulated just above the right atrial appendage with a plugged PE-190 cannula filled with saline. The heart was trimmed away by cutting just above the ventricles and placed in cold saline for subsequent determination of right ventricular hypertrophy. The lungs were removed from the thoracic cavity by gently lifting them using the tracheal and pulmonary artery cannulas and cutting along the rat's spinal column. The lungs were set in a petri dish containing saline and inflated and deflated several times to prevent atelectasis, then transferred to the perfusion apparatus.

2. 5HT uptake by IPL

For determination of 5HT uptake, lungs were perfused at 37°C in a single-pass system at a flow of 10 ml/minute using a Cole-Parmer Masterflex peristaltic pump (Cole-Parmer, Chicago, IL). The apparatus

used was similar to that of Gillis and Iwasawa (1972) and is illustrated schematically in Figure 4. The perfusion medium for these studies was Krebs-Ringer bicarbonate buffer, pH 7.4 (Table 1), aerated with 95% O_2/5% CO_2 and containing 4% bovine serum albumin (BSA; Fraction V). Inflow perfusion pressure was monitored with a Statham P23ID pressure transducer and recorded on a Grass Model 7 polygraph. The polygraph was calibrated from 0-20 mmHg using a mercury manometer. Zero pressure for the perfusion was taken as the pressure with tubing and cannulas filled with perfusion medium but without a lung in the apparatus.

After the lungs were transferred to the perfusion apparatus, they were gently inflated and deflated 20 times while being perfused to facilitate the clearance of blood from the vasculature. Two ml of room air was then injected into the tracheal cannula and it was clamped shut, keeping the lungs statically inflated. After two to three minutes of perfusion, when the inflow perfusion pressure had stabilized, the perfusion medium reservoir was switched to one containing 0.1 μM [^{14}C]-5HT. The perfusion was continued for 10 minutes and effluent samples were collected for 30 seconds beginning at 4.5 and 9.5 minutes after the switch to 5HT-containing medium. The samples were kept on ice until analyzed.

5HT was separated from its acid metabolite, 5-hydroxyindoleacetic acid (5HIAA) by cation exchange chromatography (Minard and Grant, 1972; Gillis *et al.*, 1978). Aliquots (0.5 ml) of effluent perfusion medium were applied to columns (0.5x1.5 cm) of Biorex 70 (sodium form, pH 6.0). The radioactivity in the water wash (2.5 ml)

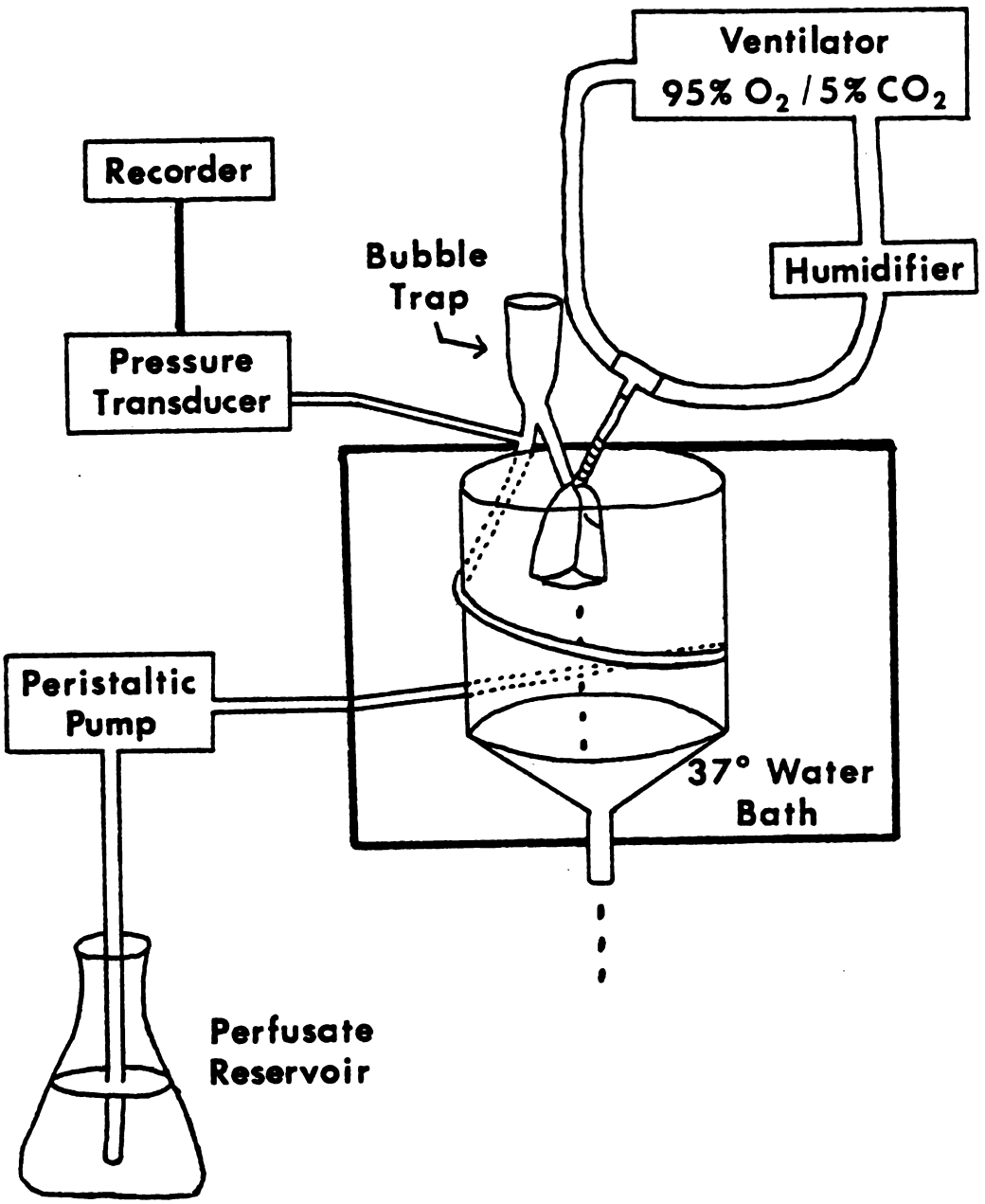


Figure 4. Diagram of isolated perfused lung preparation.

TABLE 1

Composition of Krebs-Ringer Bicarbonate Buffer

118 mM	NaCl
4.75	KCl
1.19	KH_2PO_4
1.19	$MgSO_4 \cdot 7H_2O$
2.54	$CaCl_2$
25	$NaHCO_3$
5	Glucose
Equilibrated with 95% O_2/5% CO_2	

Reference: Junod, 1972.

of this column was determined to ascertain the amount of deaminated metabolite present. Radioactivity in aliquots of the perfusion medium not subject to chromatographic separation was also determined in order to evaluate the total 5HT and 5HIAA present. Radioactivity was determined using a Beckman LSC-100 liquid scintillation counter with a window set for ^{14}C. The scintillation cocktail used was ACS^{R}. Removal of 5HT by the isolated perfused lung was calculated as the difference between the concentration of unchanged 5HT in the perfusion medium and that in the collected effluent. Percent removal (%R) of 5HT was calculated as

$$\%R = \frac{C_{a,i} - C_{a,o}}{C_{a,i}} \times 100$$

where $C_{a,i}$ and $C_{a,o}$ represent the concentrations of 5HT in the inflow and effluent perfusion medium, respectively. Similarly, the percentage of perfused 5HT in the effluent as metabolite (%M) was calculated as:

$$\%M = \frac{C_{m,o}}{C_{a,i}} \times 100$$

where $C_{m,o}$ represents the concentration of metabolite in the effluent. Values reported for %R and %M were calculated from the effluent fraction collected at 10 minutes.

3. Norepinephrine uptake by IPL

Lungs were perfused exactly as described for determination of 5HT uptake, except that the perfusion medium contained 0.1 μM [^{14}C]-norepinephrine (NE) instead of [^{14}C]-5HT. The effluent samples were collected at 1.5 and 7.5 minutes after the switch to medium

containing [^{14}C]-NE. Radioactivity in aliquots (0.5 ml) of perfusate were determined directly or after separation on Biorex 70 columns as described for 5HT. The deaminated metabolites were eluted with 2.5 ml H_2O. The unchanged amine was then removed from the columns by 3 ml 2% boric acid. O-methylated products were eluted by a final wash with 3 ml 0.2 N HCl. This method effectively separated NE (borate wash) from deaminated metabolites (H_2O wash) and metabolites that have been O-methylated but not deaminated (HCl wash) (Minard and Grant, 1972). Percent removal and metabolites were calculated as described for 5HT.

4. In vitro exposure of IPL to MCTP

To determine whether lungs could be injured by direct exposure in vitro to MCTP, the IPL protocol was slightly modified. Lungs were perfused in the same apparatus but in a recirculating system, using a reservoir volume of 50 ml KRB containing 4% BSA. Blood was cleared from the pulmonary vasculature by perfusion in a single-pass system until the inflow perfusion pressure stabilized, then the inflow cannula was switched to the recirculating reservoir. To maintain tissue oxygenation and perfusate pH, the lungs were ventilated with 95% O_2/5% CO_2 using a small animal respirator (Mallard Medical, Irvine, CA). Inspiratory pressure was 13-16 cm H_2O and a positive end expiratory pressure of 2-3 cm H_2O was maintained. The gas was humidifed by bubbling through water at 37°C en route to the lung. MCTP in DMF was infused into the bubble trap approximately 5 mm above the pulmonary artery cannula at a rate of 0.03 ml/minute using a syringe pump (Model 341, Sage Instruments, Orion Research Laboratories, Inc., Cambridge, MA). Samples were taken from the reservoir at various

times during the perfusion for determination of lactate dehydrogenase activity and MCTP concentration.

5. Vascular reactivity in the IPL

For these studies, the lungs were perfused in a cabinet maintained at 37°C (MRA Corporation, Clearwater, FL). The perfusion medium was anticoagulated whole rat blood. Male, retired breeder rats were used as blood donors. Under light ether anesthesia, the rats were bled from the abdominal aorta into syringes containing heparin. The final concentration of heparin in the perfusion medium was 30 U/ml. Reservoir volume ranged from 27-35 ml. A silk filter was inserted in the perfusate line between the pump and the bubble trap to remove any blood clots. The pH of the blood was kept between 7.35 and 7.45 by infusion of 0.1 M NH_4Cl, using a pH Controller (Horizon Ecology Company, Chicago, IL). The lungs were ventilated as described for the recirculating system above. After the lungs were suspended in the perfusion cabinet, they were perfused for 10 minutes at 10 ml/min. The flow was then increased by small increments over a period of about one minute to 20 ml/min. The preparation was allowed to stabilize another 10 minutes at this flow.

Pressure responses were measured by monitoring inflow perfusion pressure. Responses were quantified by measuring the maximum change in pressure after administration of saline, angiotensin II (AII), or 5HT. Each injection was 50 µl in volume and was injected into the perfusate line just above the pulmonary aterial cannula. Each lung received one challenge with each agent in a fixed dosage pattern: Saline, 0.125 µg AII, 12.5 µg 5HT; saline, 0.25 µg AII, 25

μg 5HT; or saline, 0.50 μg AII, 50 μg 5HT. At least 15 minutes were allowed between AII and 5HT injections for the perfusion pressure to return to baseline. In no instance did the saline injection result in a change in the perfusion pressure.

6. Dry lung weights

After all types of perfusion, the lung lobes were trimmed from the trachea and weighed in a tared glass vial. The lungs were dried in a 100°C oven until they reached constant weight.

H. Bronchopulmonary Lavage

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The trachea was cannulated with a modified 18 g needle. The bevel of the needle was filed down and about 1 cm of PE-260 tubing (Clay Adams, Parsippanny, NJ) was fitted over the tip. 500 U heparin were injected into the abdominal vena cava, and 3-5 ml of blood was withdrawn after allowing 1 minute for the heparin to distribute. The thoracic cavity was then opened and the lungs were carefully dissected free. Using a glass syringe, room temperature 0.9% saline was introduced into the airways via the tracheal cannula and immediately withdrawn. This procedure was repeated with fresh saline and the two washes were combined. The volume of saline used was the same for all animals killed on a given day. This volume was determined by multiplying the mean body weight (in kilograms) of all of the animals for that day by 23 ml/kg (Mauderly, 1977). The pooled lavage sample was centrifuged at 600 x g for 15 minutes. Blood samples were centrifuged at 10,000 x g for 10 minutes. Lactic dehydrogenase (LDH) activity in

the cell-free supernatant of the lavage fluid and in the plasma were assayed by the spectrophotometric method of Bergmeyer and Bernt (1974). This method measures LDH activity by quantifying the disappearance of the cofactor NADH using pyruvate as substrate. Protein concentrations in the cell-free lavage fluids were measured (Lowry et al., 1951) using bovine serum albumin as a standard. The cellular pellet from the lavage fluid was resuspended in 0.10 ml 0.9% saline and the cells were counted microscopically using a hemacytometer.

I. Right Ventricular Hypertrophy

The heart was kept in cold saline after removal from the rat for as brief a period as possible. The atria were trimmed from the ventricles and discarded. The right ventricle (RV) was then cut away, leaving the left ventricle plus septum (LV+S) intact. Each piece was weighed to the nearest milligram. Increases in the ratio RV/(LV+S) when the (LV+S) weight is unchanged indicate right ventricular hypertrophy (Fulton et al., 1952).

J. Platelet and White Blood Cell Counts

Aliquots of whole blood or platelet rich plasma were diluted in ammonium oxalate buffer using a Unopette^{R} diluting system (Becton Dickinson, Rutherford, NJ). Platelets and white blood cells were counted in a Neubauer hemacytometer using phase contrast microscopy (Brecher and Cronkite, 1950).

K. Preparation of Anti-Rat Platelet Antibody in the Goat

Prior to exposure to rat platelet antigen, the goat was bled to acquire 900 ml pre-immune blood from which the control serum was prepared by the same procedures as the serum containing anti-platelet antibodies. Seven days after being bled for pre-immune blood, the goat received her first innoculation with the rat platelet antigen. Her back was shaved in a strip along the spinal column and scrubbed with Betadine^{R} disinfectant solution. Rat platelet antigen (prepared as described below) was emulsified 1:1 with Freund's complete adjuvant. A total of 1 ml of the emulsification (3.25×10^{9} platelets, 4.23 mg platelet-derived protein) was injected intradermally in about 20 sites along the goat's back. Four innoculations were done at weekly intervals. Five days after the final innoculation, 30 ml of blood was collected to test for the presence of anti-rat platelet antibodies. Serum prepared from this blood caused a dramatic decrease in circulating platelet count after intravenous injection into rats. Pre-immune serum did not affect the platelet count. One week after the last innoculation, the goat was bled to acquire 900 ml blood from which anti-platelet serum was prepared.

1. Preparation of rat platelet antigen

Twenty-three male, retired breeder rats were bled under light ether anesthesia from the abdominal aorta. Blood was collected in plastic syringes containing 1 ml 3.8% sodium citrate. A total of 280 ml whole blood was collected. Platelet rich plasma (PRP) was prepared by spinning the blood at 150 x g for 5 minutes. The PRP was transferred to another tube being careful not to transfer any of the

buffy coat. The centrifugation and transfer was repeated 2 more times. The yield of PRP was 115 ml. The PRP was centrifuged 10 minutes at 150 x g to pellet the platelets. The pellet was resuspended in 3 ml 0.9% saline containing 0.01% EDTA and centrifuged again for 10 minutes at 150 x g. The supernatant was discarded and the wash repeated twice. Each pellet was resuspended in 1 ml NaCl/EDTA and all of the pellets were pooled. The final product was 10 ml of a platelet suspension containing 6.5×10^9 platelets/ml. No red or white blood cells were seen when the suspension was examined microscopically. The platelets in the suspensions were disrupted by repeated short bursts of sonication using a Sonicator Cell Disruptor (Heat Systems-Ultrasonics, Inc., Plainview, NY). The protein concentration of the sonicated mixture was 8.45 mg protein/ml. Prior to injection the antigen was emulsified with Freund's complete adjuvant in a 1:1 ratio. The emulsification was stored at 0-4°C while the remaining antigen solution was stored at -70°C.

2. Processing of goat blood

Approximately 900 ml of blood was collected from the jugular vein of the goat to prepare each batch of serum. The blood was collected in beakers and incubated at 37°C in a water bath for 2 hours to facilitate clot formation. The resultant serum was poured off and centrifuged at 750 x g for 15 minutes to remove the red blood cells. The serum was transferred to clean tubes and incubated for 45 minutes at 56°C to inactivate complement. The denatured protein was removed by centrifugation. The resulting serum was frozen at -70°C in 1 ml aliquots.

3. Absorption of serum with rat red blood cells

The antiserum was semi-purified by absorption with washed rat red blood cells (RBCs). This processing was intended to remove antibodies not specific to platelets (Friedman et al., 1977). Calling the preparation washed rat RBCs is something of a misnomer since it can contain fibrin, fibrinogen, and white blood cells as well as RBCs. By incubating the antiserum with these washed RBCs, antibodies to fibrin, fibrinogen, etc. should be removed.

Retired breeder rats were bled from the abdominal aorta under ether anesthesia into syringes containing 1 ml 3.8% sodium citrate. Platelets were removed by centrifuging 3 times at 150 x g for 5 minutes, discarding the collected PRP. The remaining cells were washed 3 times with Krebs-Ringer bicarbonate buffer (pH 7.4), discarding the washes. The absorption of the antiserum was carried out by mixing 1 part washed RBCs with 4 parts antiserum. The tubes were incubated for 2 hours at room temperature and mixed every 15 minutes. Antiserum was recovered by centrifuging 10 minutes at 600 x g. The absorption was repeated twice, using fresh, washed RBCs each time. The absorbed antiserum was frozen at -70°C in 1 ml aliquots until use. Pre-immune (control) serum was absorbed in the same manner.

L. Platelet Aggregations

1. Blood collection

Rats were lightly anesthetized with ether and bled from the abdominal aorta into 3.8% sodium citrate in plastic syringes. If necessary, more citrate was added to bring the ratio of citrate:blood to 1:9.

2. Platelet-rich plasma preparation

Blood samples were spun 3 times for 1 minute at full speed in an IEC HN-SII tabletop centrifuge. After each centrifugation the plasma layer (platelet-rich plasma, PRP) was transferred to a clean plastic tube. The remaining blood was centrifuged for 20 minutes at 2000 rpm in the IEC HN-SII centrifuge to make platelet-poor plasma (PPP). While preparing the PPP, a platelet count was done on the PRP. PPP was added to PRP in order to make the final platelet count of the PRP $1x10^6$ platelets/μl. Platelet counts were also done on the final PRP to confirm the number of platelets present.

3. Platelet aggregation

0.5 ml of PRP was pipetted into silanized glass cuvettes for aggregation measurements. The platelets were allowed to rest 45 minutes before starting the aggregations. Aggregation was measured as an increase in light transmission through a cuvette of PRP as described by Born and Cross (1963). A dual channel platelet aggregometer (Payton Associates, Buffalo, NY) was used for aggregations. Light transmission was recorded on an Omniscribe chart recorder. A cuvette containing the appropriate PPP was used to set 0 and 100% light transmission for each rat's PRP. Platelet curvettes were inserted in the aggregometer and allowed to equilibrate (37.5°C, stirred at 900 rpm) for two minutes. Three aggregating agents were tested on each rat's PRP. Adenosine diphosphate (ADP) was used at concentrations of $1x10^{-5}$M, $2x10^{-6}$M, and $1x10^{-6}$M. Collagen prepared from dog aorta was generously supplied by Dr. Thomas G. Bell of the Department of Pathology, Michigan State University. The final concentration in the PRP

was 74 μg/ml. The third aggregating agent used was arachidonic acid. Final concentrations in the PRP were 150 and 500 μg/ml. Platelet aggregation studies yielded three pieces of information for each aggregating challenge: the maximum % aggregation achieved, the rate at which aggregation occurred, and the delay between addition of aggregating agent and the response. These parameters are illustrated on a typical aggregation curve in Figure 5.

M. Drug Treatments

1. Hydralazine

Rats received 3 mg hydralazine/kg body weight, i.p., daily. Controls received 1.5 ml 0.9% saline/kg body weight. Rats were treated for 3 days prior to MCTP administration and throughout the 14 day experimental period.

2. Dexamethasone

Rats were treated with 0.027 mg dexamethasone/kg, i.p., daily. Controls received 0.5 ml 0.9% saline/kg body weight. Rats were treated for 3 days prior to MCTP administration and throughout the 14 day experimental period.

3. Sulfinpyrazone

A suspension of 100 mg sulfinpyrazone/ml was prepared by homogenizing a 200 mg Anturane^{R} capsule in 2 ml of propylene glycol. Rats were treated with 100 mg/kg, i.p., twice daily. Controls received 1 ml propylene glycol/kg using the same regimen. Rats were treated for 2 days prior to MCTP administration and throughout the 14 day experimental period.

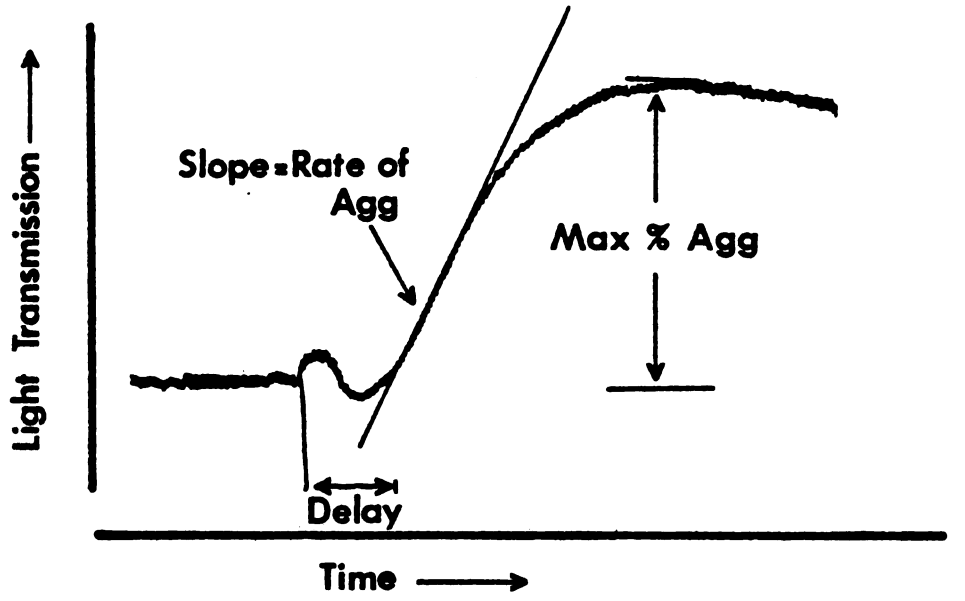


Figure 5. Platelet aggregation parameters measured. This is a tracing of the aggregation response of PRP from a DMF-treated rat to 75 μg/ml dog collagen. The slope is calculated as the rate of aggregation, expressed as % aggregation/minute. The maximum % aggregation is calculated from the maximum light transmission acheived. The delay is the time between the injection of the aggregating agent and the beginning of the aggregation response.

4. Aspirin

Rats were treated once every 5 days with 200 mg acetylsalicylic acid/kg, p.o. Acetylsalicylic acid (ASA) was suspended in 0.5% methylcellulose by teflon-glass homogenization immediately prior to administration. Controls received 4 ml 0.5% methylcellulose/kg, p.o. on the same schedule. The first ASA dose was administered 24 hours prior to MCTP treatment.

5. Metergoline

A suspension of 1 mg metergoline/ml was prepared by teflon-glass homogenization of the drug in 0.5% methylcellulose. Rats received 1 mg drug/kg or 1 ml vehicle/kg, i.p. Rats were treated 4 hours prior to MCTP administration and daily thereafter.

N. Statistical Analysis

Results are expressed as mean $\pm$ S.E.M. or mean and range, as appropriate. Student's t-test (Steel and Torrie, 1960) was used to compare means between control and treated groups in experiments having only two groups. One-way analysis of variance (completely random design) was used to evaluate differences between more than two groups (Linton and Gallo, 1975). A block factorial analysis of variance was used to analyze the effects of incubation of lung slices with MCTP *in vitro* on 5HT and PQ uptake (Linton and Gallo, 1975). Two-way factorial analysis of variance (Steel and Torrie, 1960) was used to detect differences in studies where the effect of some factor (e.g., drug treatment) on MCTP toxicity was being examined. Homogeneity of variance among the groups subjected to ANOVA was tested by the F_{max}

procedure (Steel and Torrie, 1960). The least significant difference test (Steel and Torrie, 1960) and Tukey's ω-procedure for unconfounded comparisons (Linton and Gallo, 1975) were used for individual comparisons. When the variances were nonhomogenous, logarithmic or arcsin $\sqrt{P}$ transformation of the data was performed. If the variances remained nonhomogenous, the preplanned individual comparisons were made using the nonparametric rank sum test (Hollander and Wolfe, 1973). In all cases, the criterion for significance was $p<0.05$. Linear regression analysis was used to construct standard curves for the protein assay and the Ehrlich assay for pyrroles (Steel and Torrie, 1960).

O. Chemicals

Monocrotaline was obtained from S.B. Penick and Co., Lyndhurst, NJ and Trans World Chemicals, Washington, DC. Adam's catalyst (Platinum (IV) oxide) was purchased from Aldrich Chemical Company, Milwaukee, WI. Bovine serum albumin (Fraction V) was purchased from Miles Biochemicals, Elkhart, IN. Each lot of BSA was tested for MAO activity using ^{14}C-5HT as substrate. Biorex 70 cation exchange resin is a product of Biorad Laboratories, Richomond, CA. Freund's complete adjuvant was obtained from Grand Island Biological Company, Grand Island, NY.

The drugs used in this study came from a variety of sources. Sodium phenobarbital and sodium pentobarbital were purchased from Ganes Chemicals, Pennsville, NJ. Dexamethasone (Azium^{R}, Schering Corporation, Kenilworth, NJ) and sulfinpyrazone (Anturane^{R}, CIBA

Pharmaceutical Company, Summit, NJ) were purchased through the MSU Clinical Center pharmacies. Angiotensin II amide was obtained from CIBA Pharmaceutical Company, Summit, NJ. Hydralazine hydrochloride (Apresoline^{R}) was a generous gift from the CIBA Pharmaceutical Company. Other drugs used were obtained from Sigma Chemical Company, St. Louis, MO (acetylsalicylic acid, imipramine, iproniazid and heparin).

Arachidonic acid for platelet aggregation studies was obtained from Bio/Data Corporation, Hatboro, PA. Dog collagen was the kind gift of Dr. T.G. Bell, Michigan State University, Department of Pathology. Adenosine diphosphate was purchased from Sigma Chemical Company, St. Louis, MO. Prosil 28^{R}, a silanizing agent for glassware, was obtained from PCR Research Chemicals, Gainesville, FL.

Radiolabelled chemicals were obtained from Amersham, Arlington Heights, IL. 5-hydroxy[side chain-2-^{14}C]tryptamine creatinine sulfate had a specific activity of 58 mCi/mmol. dl-[8-^{14}C]noradrenaline dl-bitartrate had a specific activity of 55 mCi/mmol. Paraquat chloride, labelled with a methyl-^{14}C, had a specific activity of 111 mCi/mmol. Scintillation cocktails used were ACS^{R} (Amersham) and 3a20^{R} (Research Products International, Mount Prospect, IL). Tissues were solubilized in Soluene 350^{R} from Packard Instrument Company, Downer's Grove, IL.

Sigma Chemical Company, St. Louis, MO, was the supplier of p-dimethylaminobenzaldehyde, 14% boron trifluoride in methanol, paraquat (methyl viologen), 5HT creatinine sulfate complex, bovine serum albumin for standardization of the Lowry protein assay, NADH, methylcellulose and sodium pyruvate solution. Kits for the determination of serum glutamic-oxalacetic and glutamic-pyruvic transaminases, blood urea nitrogen and creatinine were also obtained from this source.

Neutral alumina was purchased from Biorad Laboratories, Richmond, CA and Fisher Scientific Company, Fairlawn, NJ. Molecular sieves to maintain the dryness of the ultra-dry methanol also came from Fisher. Absolute ethanol (USP, 200 proof) was obtained from Aaper Alcohol and Chemical Company, Louisville, KY. The remaining chemicals used were purchased from Mallinckrodt, Inc., Paris, KY. These were: 30% hydrogen peroxide, anhydrous sodium sulfate and ethyl ether, N,N-dimethylformamide, ammonium chloride, magnesium sulfate, glucose, sodium bicarbonate, sodium chloride, potassium chloride, mono- and di-basic potassium phosphate, potassium sodium tartrate, cupric sulfate, ferrous sulfate, sodium citrate, sodium fluoride, ascorbic acid, iodine and acetone.

RESULTS

A. Dose Response to a Single Injection of MCT

A single subcutaneous injection of MCT at a dose of 60 mg/kg results in pulmonary hypertension between three and six weeks after injection (Hayashi and Lalich, 1967). The purpose of these experiments was to identify a dose of MCT which would result in reproducible pulmonary hypertension and other lung effects within 2-3 weeks after a single injection.

Three subcutaneous doses were examined: 60 mg MCT/kg, 105 mg MCT/kg, and 130 mg MCT/kg. Figure 6 shows the effects of these doses on the ratio of right ventricular weight to left ventricle plus septum weight (RV/LV+S). An increase in RV/(LV+S) reflects right ventricular hypertrophy (Fulton et al., 1952). No right ventricular hypertrophy was evident 10 days after any of the doses administered. By 14 days, however, the relative weight of the right ventricle had increased in rats given 105 or 130 mg MCT/kg. A decrease in (LV+S) weight contributed to the increased ratio for 130 mg/kg, 14 day data. The ratio of (LV+S)/BW was decreased in rats surviving 130 mg MCT/kg and the ratio of RV/BW was not elevated. For all of the other groups (LV+S)/BW was unaffected by MCT treatment. The mortality in the group receiving 130 mg/kg was 35%. Right ventricular weight was assessed only in the surviving animals, perhaps explaining why the right ventricular hypertrophy was less marked in this group.

Figure 6. Effect of MCT dose on relative right ventricular weight. Rats were treated with indicated doses of MCT (s.c.) or with 0.9% saline and the ratio of right ventricle weight (RV) to the weight of left ventricle plus septum (LV+S) was determined 10 or 14 days later. Bars indicate mean $\pm$ SEM of groups of 6-8 rats. Hatched bars - saline, open bars - MCT. *Significant difference from control group (Student's t-test, $p<0.05$).

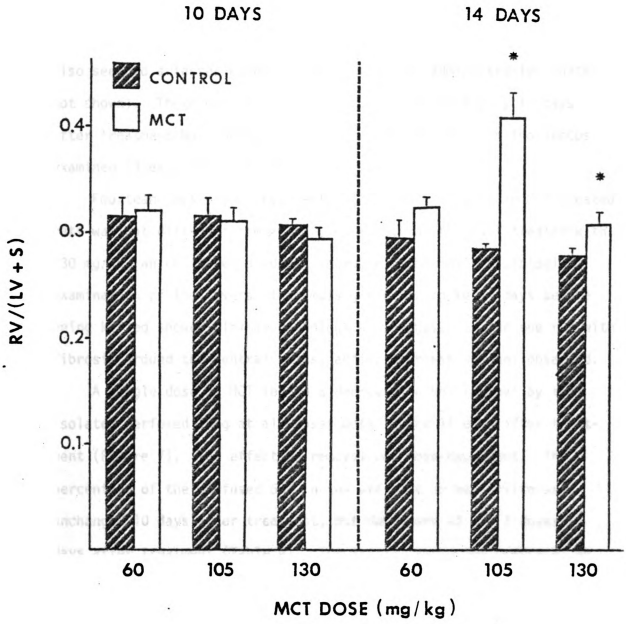

10 DAYS
14 DAYS
CONTROL
MCT
0.4
0.3
0.2
0.1
RV/(LV + S)
*
*
60
105
130
60
105
130
MCT DOSE (mg/kg)

Figure 6.

The effects of MCT on body weight gain and lung weight after 14 days are summarized in Table 2. MCT treatment decreased the body weight gain and increased the wet lung weight. Absolute dry lung weight was increased only in the 105 mg/kg group. These effects were also seen to a lesser extent 10 days after MCT administration (data not shown). There was no mortality at any dose tested by 10 days after treatment but the mortality increased with dose in the groups examined 14 days after treatment.

Fourteen days after treatment, plasma GPT activity in MCT-treated rats was not different from controls except for the rats treated with 130 mg/kg, which showed a modest increase (Table 3). Histological examination of the livers of animals given 105 mg/kg 14 days before being killed showed minimal pathological changes. In the one rat with fibrosis around the central veins, active necrosis was not observed.

A single dose of MCT led to a decrease in 5HT removal by the isolated perfused lung at all doses both 10 and 14 days after treatment (Figure 7). The effect on removal was dose-dependent. The percentage of the perfused 5HT in the effluent as metabolite was unchanged 10 days after treatment, but decreased at all 3 doses 14 days after treatment (Table 4). The initial perfusion pressure, an indication of pulmonary vascular resistance, was also unchanged at 10 days but was elevated after 14 days in the groups treated with 60 or 105 mg/kg (Table 4).

TABLE 2

Body Weight Gain, Lung Weight and Mortality 14 Days After MCT Injection

	Treatment[a]					
	Saline	60 mg/kg	Saline	105 mg/kg	Saline	130 mg/kg
ΔBW (g)	100±3 (7)	55±3 (7)	102±3 (8)	17±14[b] (7)	83±4 (6)	-30±10[b] (6)
Wet Lung (g)	1.692±0.073 (6)	2.156±0.088[b] (6)	1.877±0.118 (7)	3.488±0.392[b] (7)	1.372±0.036 (6)	2.180±0.394 (6)
Wet/Dry Lung Weight	6.4±0.3 (6)	7.2±0.2[b] (6)	7.4±0.3 (7)	8.1±0.3 (7)	5.5±0.1 (6)	8.0±0.9[b] (6)
Mortality	0/7	0/7	0/8	1/8	0/8	4/11

[a]Rats were given a single subcutaneous injection of MCT or an equivalent volume of 0.9% ssaline 14 days before sacrifice. "ΔBW" is the difference between the body weight at 14 days and the body weight on the day of MCT treatment. Mortality is expressed as the number of rats dying before the end of the 14 day treatment period over the number treated. All other values represent mean ± S.E.M. with the number of rats in each group indicated in parentheses.

[b]Significantly different from appropriate saline control group (Student's t-test, $p<0.05$).

TABLE 3

Effect of MCT Injection on Plasma Glutamate Pyruvate Transaminase

Days After Treatment	Treatment[a]					
	Control	60 mg/kg	Control	105 mg/kg	Control	130 mg/kg
10 days	13.8±1.3 (6)	12.5±1.2 (6)	13.8±1.3 (6)	17.9±1.4 (7)	9.8±2.8 (8)	13.6±4.1 (8)
14 days	14.8±4.1 (7)	13.2±3.1 (7)	17.7±1.2 (8)	17.6±2.9 (7)	14.9±0.8 (6)	30.3±3.9 (6)[b]

[a]Rats were given a single subcutaneous injection of MCT or an equivalent volume of 0.9% saline. Plasma GPT activities were measured as described in "Materials and Methods". Values are reported in Sigma-Frankel units/ml as mean ± S.E.M. The number of animals in each group is indicated in parentheses.

[b]Significantly different from control group (Student's t-test, $p<0.05$).

Figure 7. Effect of MCT dose on 5HT removal by the isolated, perfused lung. Rats were treated with indicated doses of MCT (s.c.) or 0.9% saline and 5HT removal was determined 10 or 14 days later as described in "Materials and Methods". Bars indicate mean $\pm$ SEM for groups of 4-8 rats. Open bars - saline, hatched bars - MCT. *Significant difference from control group (Student's t-test, $p<0.05$).

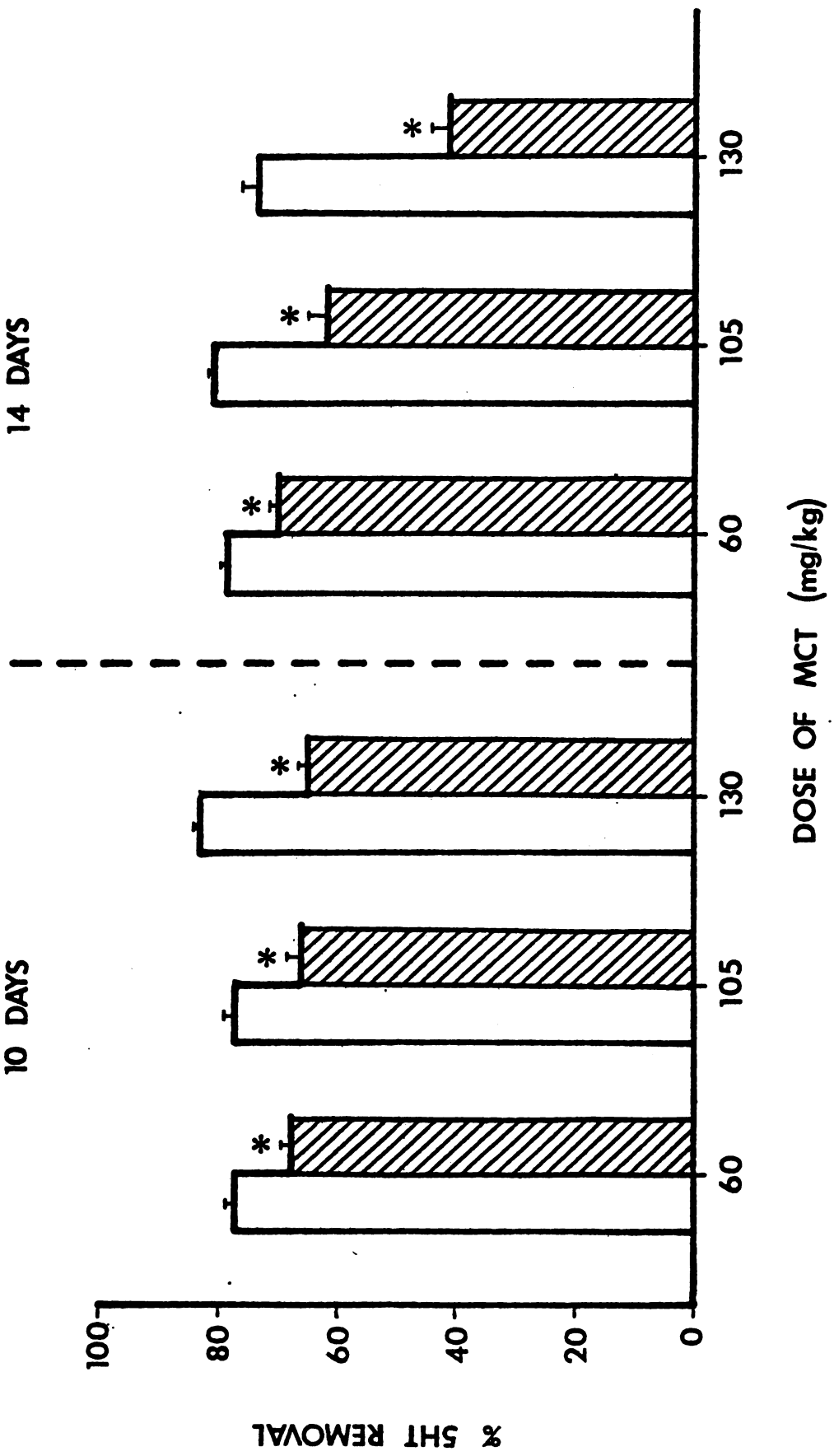

% 5HT REMOVAL
100
80
60
40
20
0
10 DAYS
14 DAYS
60
105
130
130
105
60
DOSE OF MCT (mg/kg)
*

Figure 7.

TABLE 4

Effect of MCT Injection on Perfused Lung Parameters

Parameters	Days After Treatment	Treatment[a]					
		Saline	60 mg/kg	Saline	105 mg/kg	Saline	130 mg/kg
% 5HT Metabolites	10	41.1±1.8 (5)	37.8±1.6 (4)	41.1±1.8 (5)	37.7±2.0 (7)	49.1±1.7 (8)	35.9±1.1[b] (8)
	14	53.1±0.9 (6)	47.4±0.7[b] (6)	55.3±1.0 (6)	36.6±2.1[b] (5)	47.6±0.5 (5)	28.7±1.9[b] (6)
Initial Perfusion Pressure (mmHg)	10	8±2 (5)	8±0 (4)	8±2 (5)	8±1 (7)	7±0 (8)	10±1[b] (8)
	14	6±0 (6)	10±1[b] (6)	7±0 (6)	9±1[b] (7)	6±2 (5)	9±3 (6)

[a]Rats were given a single subcutaneous injection of MCT or an equivalent volume of 0.9% saline. "% 5HT Metabolites" expresses the percentage of perfused 5HT emerging in the effluent as 5HT metabolites. The perfusion procedure is described in detail in "Materials and Methods". Values are reported as mean ± S.E.M. The number of animals in each group is indicated in parentheses.

[b]Significantly different from corresponding saline control group (Student's t-test, $p<0.05$).

B. Development of Responses to a Single Injection of MCT

A dose of 105 mg MCT/kg (s.c.) was chosen for further characterization because it caused right ventricular hypertrophy within 14 days of administration with minimal mortality. Rats treated with 105 mg MCT/kg gained weight more slowly than the corresponding controls (Table 5). By 5 days after MCT treatment wet lung weights from the MCT-treated rats were increased and remained so at 10 and 14 days (Table 5). Dry lung weights were elevated only 14 days after treatment. Wet/dry lung weight ratios were unchanged at any of the time points examined.

A single injection of MCT caused a progressive decrease in the removal of 5HT by the isolated, perfused lung. Figure 8 shows the time course of this effect. There was no difference between the saline- and MCT-treated animals 2 days after injection, but at 5, 10 and 14 days after treatment, 5HT removal was significantly decreased (11, 15 and 23% below control, respectively). The percentage of the perfused 5HT appearing as metabolite in the effluent perfusion medium is shown in Table 6. The percent metabolite was decreased at 5 and 14 days after treatment. Inflow perfusion pressure at the beginning of the 5HT perfusion was increased in lungs of MCT rats only at 14 days after treatment (saline, 7 ± 0 mmHg; MCT, 9 ± 0 mmHg). Inflow perfusion pressure did not change between the beginning and the end of the perfusion for any of the control or treated animals.

Monocrotaline treatment caused a significant decrease in the circulating platelet count (Figure 9). At 2 and 5 days after treatment, the platelet count in MCT animals was less than 45% of that in control rats. Ten days after treatment, the count was still

TABLE 5

Effects of 105 mg MCT/kg on Body Weight Gain and Lung Weights[a]

		Days After Treatment			
		2	5	10	14
ΔBW (g)	Saline	8±2 (8)	33±3[b] (8)	62±2[b] (6)	102± 3[b] (8)
	MCT	-3±3[b] (8)	-12±5[b] (9)	12±4[b] (7)	17±14[b] (7)
Wet Lung (g)	Saline	1.57±0.04 (8)	1.54±0.04[b] (8)	1.35±0.04[b] (6)	1.88±0.12[b] (7)
	MCT	1.56±0.05 (8)	1.73±0.05[b] (8)	1.86±0.20[b] (7)	3.49±0.39[b] (7)
Wet/Dry Lung Weight	Saline	7.7±0.2 (8)	7.2±0.2 (8)	6.2±0.1 (6)	7.4±0.3 (7)
	MCT	7.7±0.2 (8)	7.6±0.2 (9)	6.4±0.1 (7)	8.1±0.3 (7)

[a]Rats were treated with 105 mg MCT/kg or an equivalent volume of saline, s.c. and killed at the indicated periods after injection. "ΔBW" is the difference between weight on the day of sacrifice and initial body weight. Lungs were weighed after perfusion. Values reported are mean ± S.E.M., with the number of animals per group in parentheses.

[b]Significantly different from corresponding saline group (Student's t-test, $p<0.05$).

Figure 8. Development of MCT effects on 5HT removal by the isolated, perfused lung. Rats were treated with 105 mg MCT/kg (s.c.) or with 0.9% saline. 5HT removal was determined at various times after injection as described in "Materials and Methods". Points represent mean ± SEM of groups of 5-9 rats. *Significant difference from control group of same day (Student's t-test, $p<0.05$).

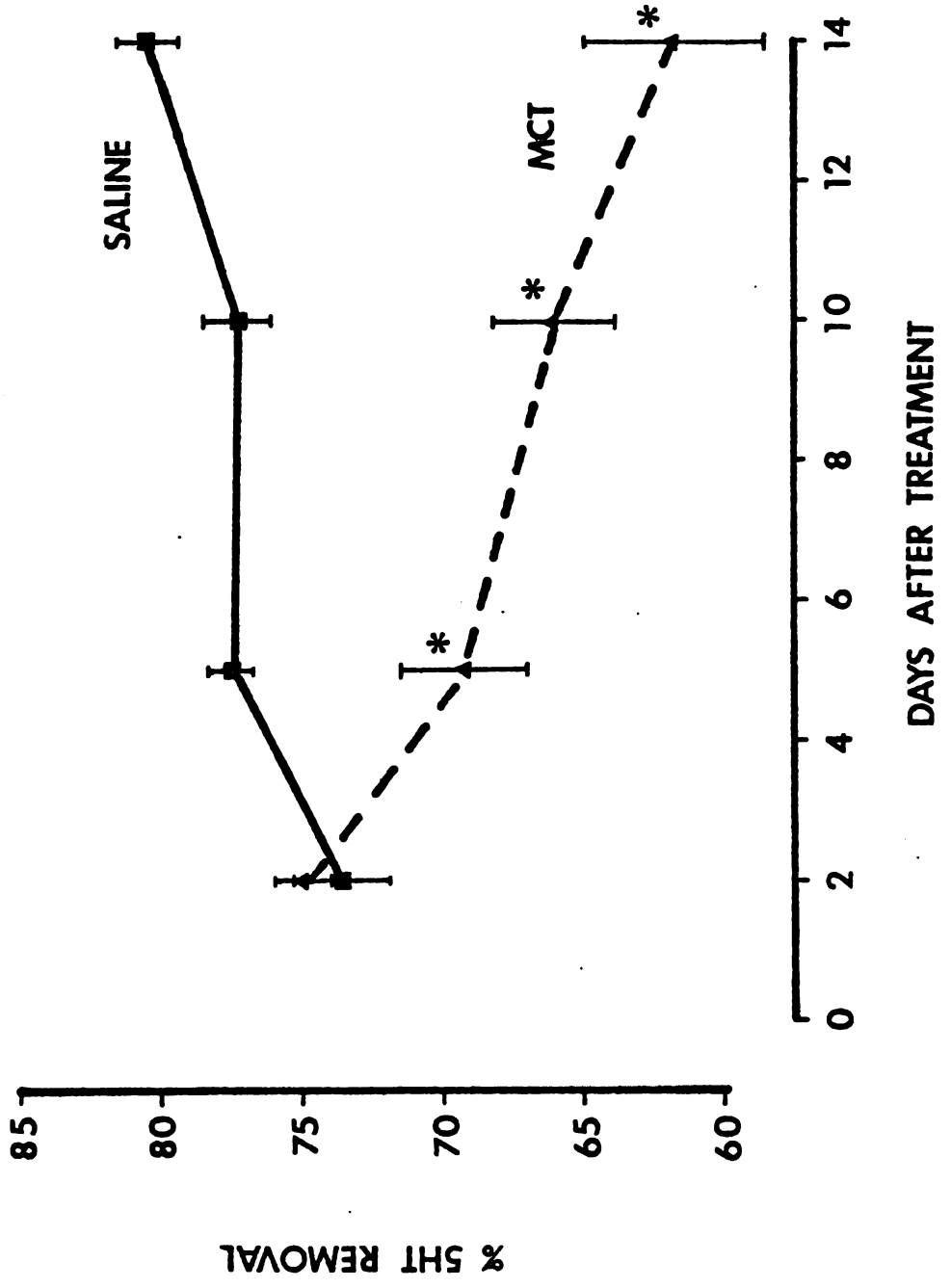

SALINE
MCT
*
*
*
*
85
80
75
70
65
60
0
2
4
6
8
10
12
14
% 5HT REMOVAL
DAYS AFTER TREATMENT

Figure 8.

TABLE 6

Effect of 105 mg MCT/kg on Isolated, Perfused Lung Parameters[a]

		Days After Treatment			
		2	5	10	14
% 5HT Metabolites	Saline	49.0±2.0	50.9±0.7[b]	41.1±1.8	55.3±1.0[b]
	MCT	49.4±1.6	44.7±1.2[b]	37.7±2.0	36.6±2.1[b]
Initial Perfusion Pressure (mmHg)	Saline	7±0	6±0	8±0	7±0[b]
	MCT	7±0	7±0	8±0	9±0[b]

[a]Rats were treated with 105 mg MCT/kg or an equivalent volume of saline, s.c., and killed at the indicated periods after injection. "% 5HT Metabolites" expresses the percentage of perfused 5HT emerging in the effluent as 5HT metabolites. The perfusion procedure is described in detail in "Materials and Methods". Values reported as mean ± S.E.M. N = 5-9 per group.

[b]Significantly different from corresponding saline control group (Student's t-test, $p<0.05$).

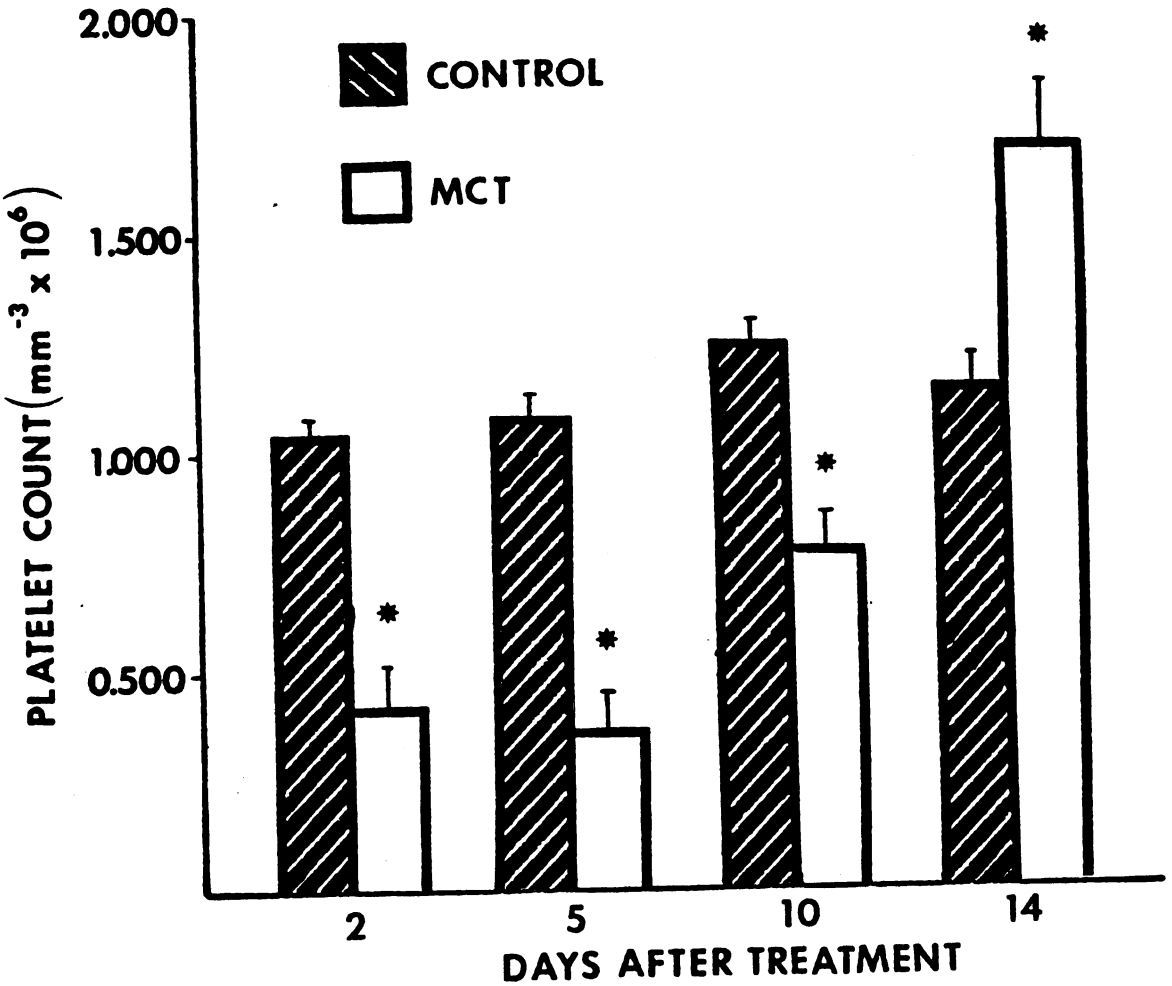


Figure 9. Effects of MCT on circulating platelet count at various times after injection. Rats were treated with 105 mg MCT/kg or 0.9% saline, s.c. Platelets were counted in venous blood obtained during surgical procedures preceding lung perfusion. Bars are mean ± SEM of groups of 6-9 rats. Hatched bars- saline, open bars- MCT. * Significant difference from control groups of same day (Student's t-test, $p < 0.05$).

significantly decreased but to a lesser extent. By 14 days, however, the platelet count in the treated animals was 47% greater than controls. The platelet counts of the control animals compare favorably with the normal range of 873,000-1,229,000/mm^3 for the rat (Schalm, 1975).

Plasma glutamate pyruvate transaminase (GPT) activity in the MCT animals was significantly increased 2 days after treatment but did not differ from controls at any of the subsequent time points (Figure 10).

Right ventricular hypertrophy as measured by an increase in the RV/(LV+S) ratio was not evident until 14 days after MCT treatment (Figure 11).

Although the development of the effect was not investigated, a separate set of animals was treated with 105 mg MCT/kg to determine whether norepinephrine (NE) removal and metabolism were also altered by MCT. These results are presented in Table 7. Removal of NE by the isolated, perfused lung was decreased 26% fourteen days after MCT administration. The percentage of perfused NE in the effluent as metabolites was not changed. The perfusion pressure rose slightly over the course of the perfusions in both control and treated lungs. There was no difference in the magnitude of the rise between the two groups, however.

C. Synthesis of MCTP

The product of the synthesis procedure described in the "Materials and Methods" section is a white, crystalline solid that is sensitive to acid, moisture, oxygen and heat. Exposing the product to any of

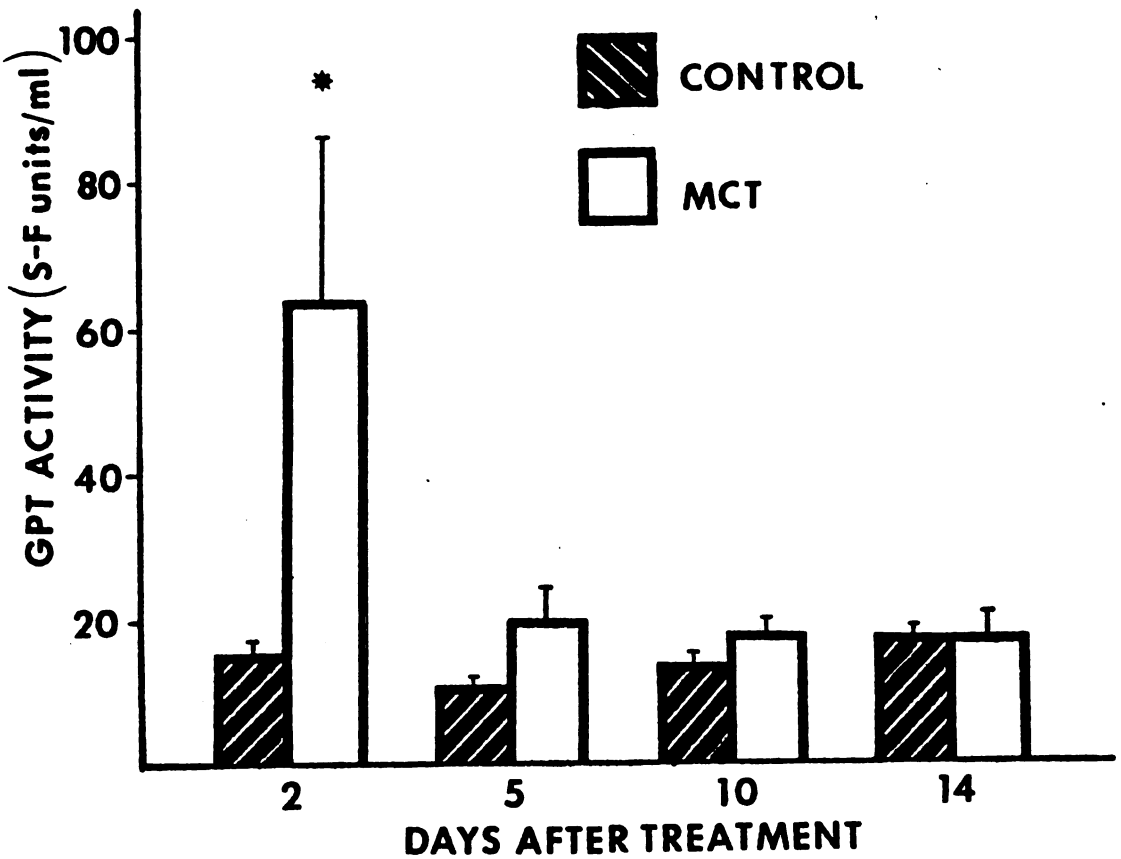


Figure 10. Effects of MCT on plasma glutamic pyruvic transaminase activity. Rats were treated with 105 mg MCT/kg or 0.9% saline, s.c. GPT activity is reported as Sigma-Frankel units/ml plasma. Bars are mean + SEM of groups of 6-9 rats. Hatched bars- saline, open bars- MCT. * Significant difference from control of same day (Student's t-test, $p < 0.05$).

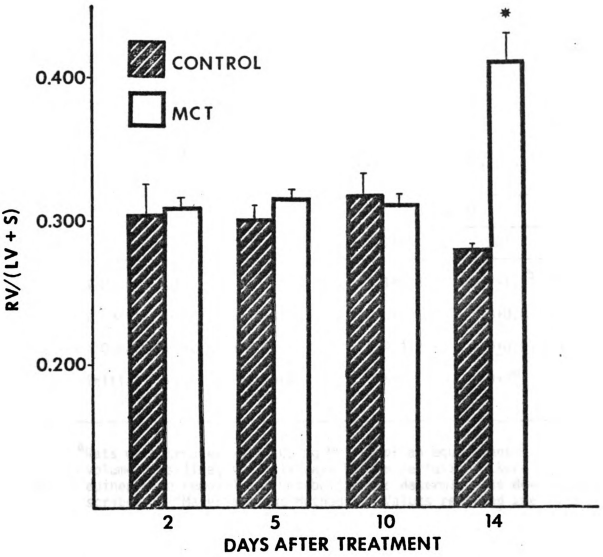


Figure 11. Development of right ventricular hypertrophy after MCT administration. Rats were treated with 105 mg MCT/kg or 0.9% saline, s.c., at the indicated times before sacrifice. RV/(LV+S) is the ratio of right ventricle to left ventricle plus septum weight. Bars are mean $\pm$ SEM of groups of 6-9 rats. Hatched bars- saline, open bars- MCT. * Significant difference from control of same day (Student's t-test, $p < 0.05$).

TABLE 7

Effect of MCT on Norepinephrine Removal and Metabolism in the Isolated, Perfused Lung[a]

	Treatment	
	Saline	MCT
% NE Removal	21.1±1.8	15.7±1.3[b]
% Deaminated Metabolites	9.8±1.1	8.3±0.8
% O-methylated Metabolites	9.4±4.2	3.9±0.9
Initial Perfusion Pressure (mmHg)	6±1	9±1[b]

[a]Rats were treated with 105 mg MCT/kg or an equivalent volume of saline, s.c., 14 days before perfusion. Norepinephrine removal and metabolism was determined as described in "Materials and Methods". Values reported are mean ± S.E.M. N = 12 for the saline group and N = 16 for the MCT group.

[b]Significantly different from saline group (Student's t-test, $p<0.05$).

these stresses results in a progressive change from white crystals to an orange/brown tar. The product decomposes at temperatures between 90 and 100°C. It is soluble in diethyl ether, dimethylformamide, bis-2-methoxyethyl ether (diglyme), and dimethylsulfoxide. It is only sparingly soluble in chloroform, acetone or toluene. It decomposes in ethanol, methanol, water and acids. The product contains a pyrrole moiety because it will react in a concentration dependent fashion with Ehrlich's reagent (Figure 12).

Biologically, the product produces lung lesions after tail vein injection of small doses. The doses and lesions are comparable to those previously reported (Butler et al., 1970; Chesney et al., 1974). These results are discussed in more detail in the next section. Batch to batch variation in the severity of the lesions and the mortality by 14 days after treatment were noted occasionally, but could not be correlated with visible differences in the pyrrole or any of the variables in the synthesis procedures.

Mass spectrometry of the product confirmed that it contains some dehydromonocrotaline. Figure 13 is the mass spectrum of one batch of MCTP. The major ion fragments reported in the literature for MCTP are: m/e 323, 250, 234, 119, 118, 117, 99, 97, 89, 83, 78, 73, 71, 69 (Culvenor et al., 1970) and m/e 323, 250, 234, 206, 191, 170, 143, 135, 126, 120, 119, 118, 117, 116 (Mattocks, 1969). The major fragments observed in our spectra compare favorably with these. Analysis of several batches of MCTP suggested that an impurity existed in our product because there were indications of at least two compounds in

Figure 12. Standard curve for Ehrlich assay. Points represent 3 separate standard curve determinations performed as described in "Materials and Methods". The line was drawn using linear regression analysis.

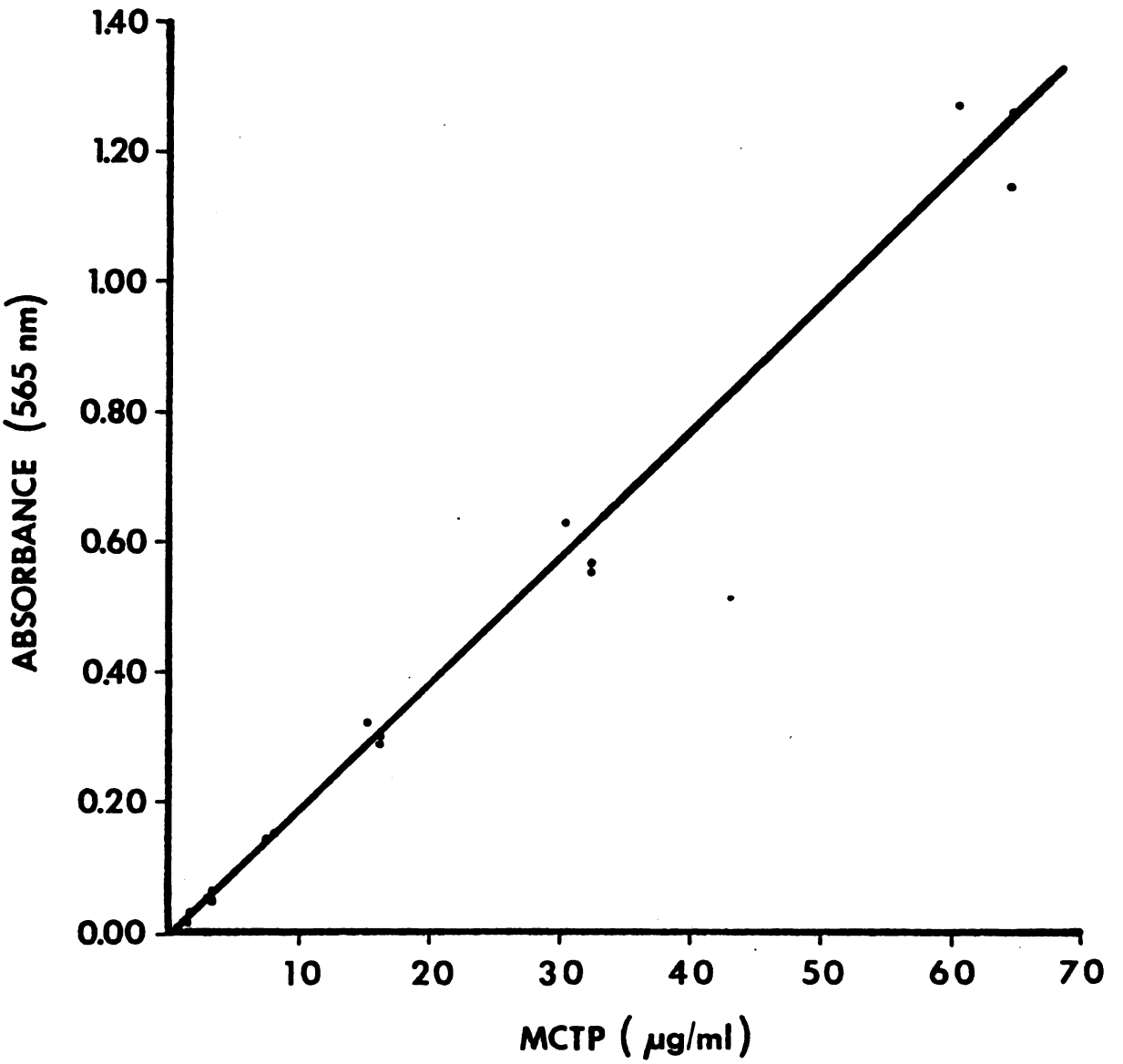

1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
ABSORBANCE (565 nm)
10
20
30
40
50
60
70
MCTP (µg/ml)

Figure 12.

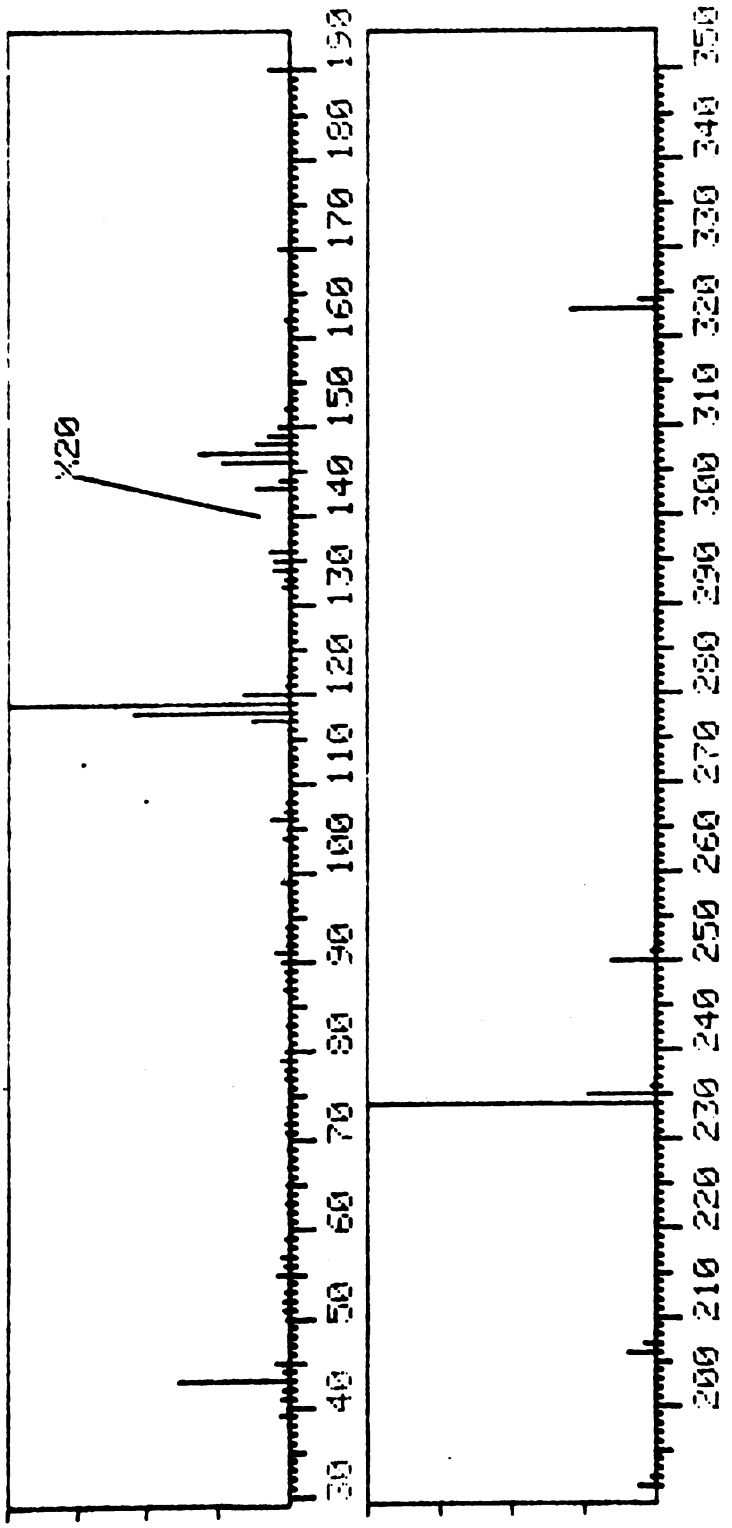


Figure 13. Mass spectrum of MCTP. This spectrum is the result of direct probe electron impact mass spectrometric analysis. Accelerating voltage was 70 eV. The temperature was increased from 50-300°C at approximately 100°C/minute. The ordinate represents the relative abundance of each fragment and the abcissa indicates the m/e ratio. The relative abundance was multiplied by a factor of 20 from m/e 140 onward to improve recognition of ion fragments.

the ion current for each sample. The impurity did not have the same fragmentation pattern from batch to batch.

Thin layer chromatography (TLC) was used in an attempt to isolate and quantify the impurity(ies) in the batches of MCTP. Development of TLC plates in the solvent system described in the "Materials and Methods" section (diethyl ether, methanol, diethylamine) provided the best separation of the various solvent systems examined. In this system, when the plate was developed with Ehrlich's reagent, four to six spots were typically observed. Figure 14 is a diagram of a TLC plate developed in this manner. The spots had R_f values as follows: A-0.96, B-0.65, C-0.51, D-0.47, E-0.20. Spot D, the most prominent, was assumed to be the desired product, MCTP. To determine whether this was the case, a preparatory plate was run. A band of gel at the position of spot D (before spraying with Ehrlich's reagent) was scraped from the plate. The compound composing spot D was then eluted with the same solvent used to develop the plate. The eluate was filtered and concentrated and a direct probe mass spectrometric analysis was performed. The compound had a mass ion fragment of 323 m/e and a fragment at 234, consistent with the structure of MCTP.

The presence of 5 spots on the chromatogram of a supposedly pure compound was not encouraging. However, given the pyrrole moiety's reactivity it seemed possible that the compound was breaking down in the course of the analysis. To test this hypothesis, a preparatory plate was developed and the presumptive MCTP spot was eluted, filtered and concentrated as before. The eluate was then spotted on a fresh TLC plate and developed. This yielded a plate with four spots with R_f

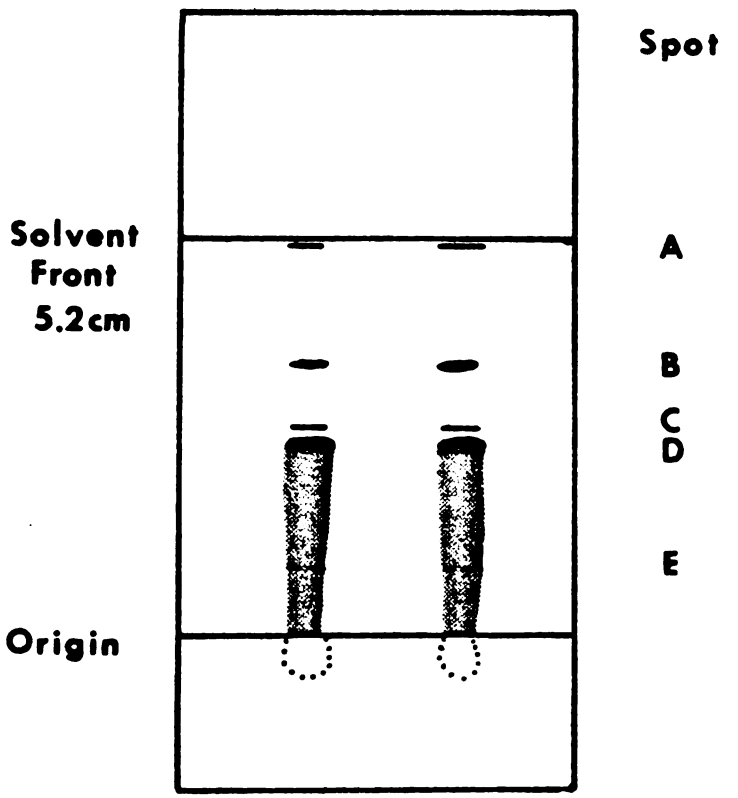


Figure 14. Diagram of TLC separation of MCTP product. Each channel was spotted with 10 μl of 2 mg MCTP/ml in dried diethyl ether. The plate was developed in diethyl ether: methanol : diethylamine (96.8 : 2 : 1.2). Spots were detected by spraying with Ehrlich's reagent followed by heating.

values close to those observed after spotting the original synthesis product. Since the eluate contained only one compound in theory, and four were detected on the plate, it was likely that some aspect of the TLC procedure was causing the product to break down. Hence, this particular TLC system is not suitable for determinations of purity. Attempts to create a system which separates the product into more than one component but does not catalyze the formation of new components have been unsuccessful to date.

Overall, the synthesis procedure yields a product with many of the chemical characteristics and biological activities attributed to dehydromonocrotaline. However, the exact chemical composition and purity of our product are not known.

D. Development of Responses to a Single Injection of MCTP

A dose of 5 mg MCTP/kg, i.v., results in pulmonary hypertension within two weeks as indicated by an increase in RV/(LV+S) 14 days after treatment (Figure 15). Other studies have shown that right ventricular hypertrophy has not developed by 10 days after MCTP administration (Bruner et al., 1983).

As observed after MCT treatment, MCTP slowed the weight gain of treated rats examined 5, 7 and 14 days after treatment. Wet lung weights were increased in the treated rats at all three time points (Table 8). Dry lung weights were also significantly elevated at all three times by MCTP treatment. The ratio of wet/dry lung weights was increased at 5 and 14 days after MCTP treatment.

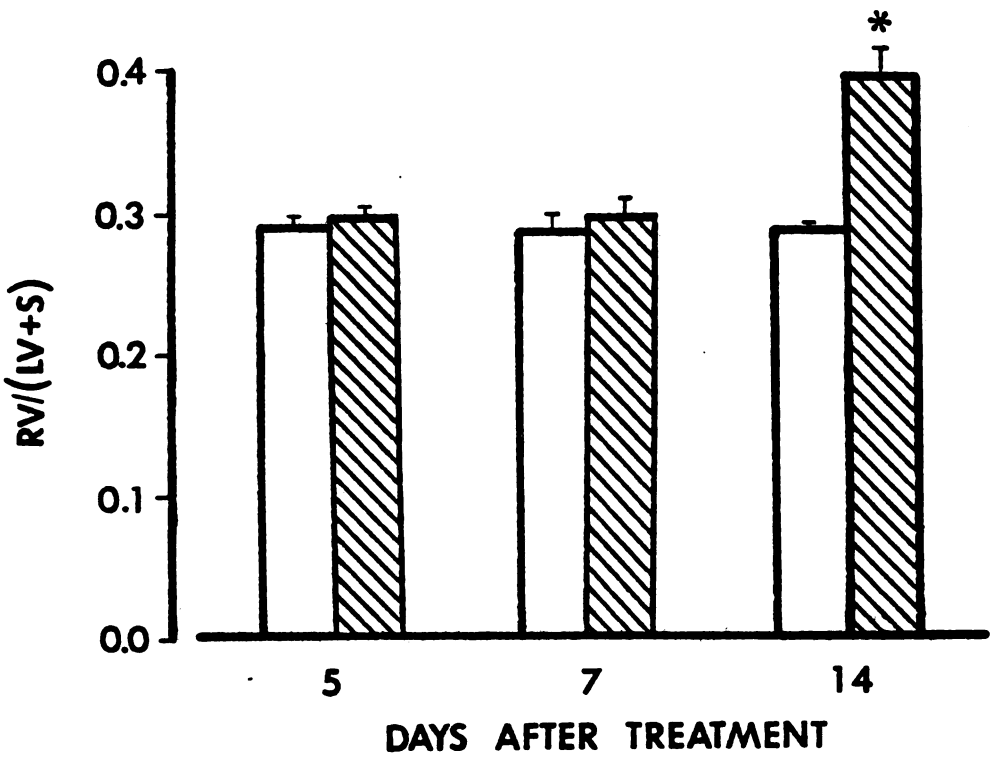


Figure 15. Development of right ventricular hypertrophy after MCTP administration. Rats were treated with 5 mg MCTP/kg or 0.5 ml DMF/kg, i.v., at the indicated times before sacrifice. RV/(LV+S) is the ratio of right ventricle to left ventricle plus septum weight. Bars are mean ± SEM of groups of 7-13 rats. Open bars- DMF, hatched bars- MCTP. * Significant difference from DMF group of same day (Student's t-test, $p < 0.05$).

TABLE 8

Effect of MCTP on Body and Lung Weights[a]

		Days After Treatment		
		5	7	14
ΔBW (g)	DMF	36±2 (13)	47±2 (14)	87± 3 (7)
	MCTP	16±2[b] (13)	32±3[b] (16)	-11±14[c] (6)
Wet Lung Weight (g)	DMF	1.217±0.059 (13)	1.157±0.043 (14)	1.181±0.043 (7)
	MCTP	1.592±0.095[b] (13)	1.712±0.091[b] (16)	2.581±0.307[b] (6)
Wet/Dry Lung Weight	DMF	5.9±0.1 (13)	5.8±0.1 (13)	5.2±0.1 (7)
	MCTP	6.5±0.3[b] (13)	6.3±0.2 (16)	7.4±0.6[b] (6)

[a]Rats were treated with 0.5 ml DMF/kg or 5 mg MCTP/kg, i.v., and sacrificed 5, 7, or 14 days later. "ΔBW" is the difference in body weight between the day of sacrifice and the day of treatment. Wet lung weights were determined after perfusion as described in "Materials and Methods". Values reported are mean ± S.E.M. with the number per group in parentheses.

[b]Significantly different from corresponding DMF group (Student's t-test, $p<0.05$).

[c]Significantly different from corresponding DMF group (Rank sum test, $p<0.05$).

In contrast to the effects of MCT, 5HT removal was not one of the earliest changes seen after MCTP (Figure 16). At 5 and 7 days after treatment the percentage of perfused 5HT removed by the pulmonary vasculature was no different from that seen in DMF-treated animals. Fourteen days after treatment, 5HT removal by MCTP-treated lungs was decreased by 25% relative to the control lungs. The percentage of perfused 5HT in the effluent as metabolite was significantly decreased at all three times examined (Figure 17). Initial perfusion pressure was not elevated in any of the treated groups.

MCT and MCTP also had very different effects on the circulating platelet count (Figure 18). In contrast to MCT, MCTP did not cause a decrease in the platelet counts at any time between 30 minutes and 14 days after administration.

E. In Vitro Exposure of Lung Slices to MCT Metabolites

These experiments were undertaken with two major objectives: (1) To determine whether exposure of lung slices directly to MCT metabolites *in vitro* would alter the ability of the slices to accumulate 5HT and PQ and (2) to address a criticism of the isolated perfused lung technique by determining whether 5HT uptake in slices is altered to the same extent as 5HT removal in the isolated perfused lung by pretreatment of the animal with MCT or MCTP. In the perfused lung, depressed 5HT removal due to altered cellular uptake of 5HT cannot be distinguished from that due to alterations in distribution of perfusion.

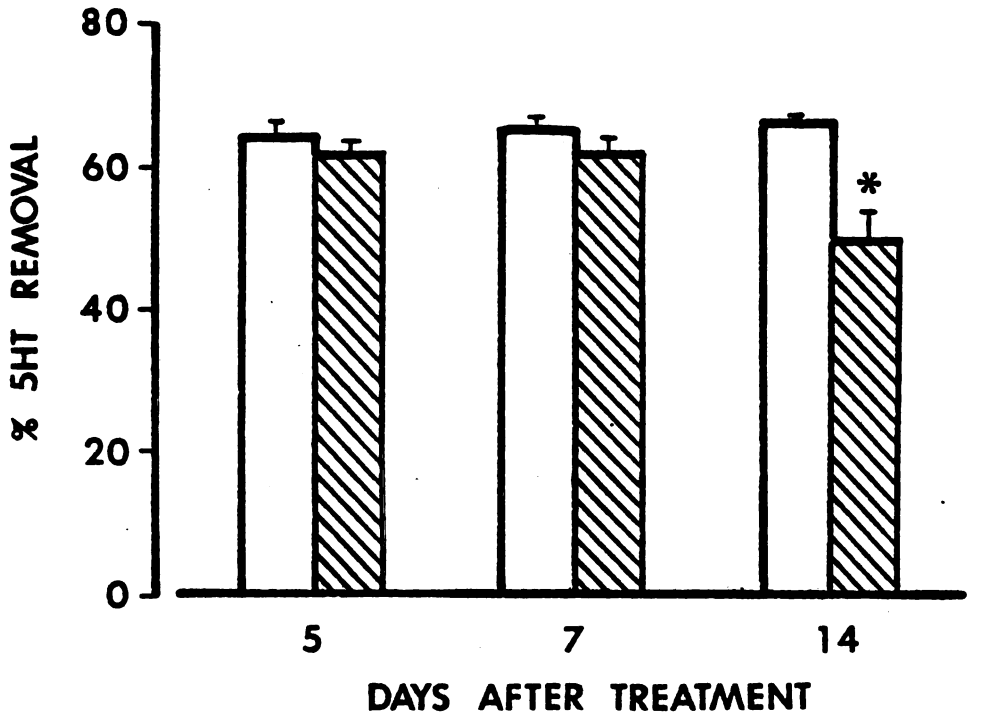


Figure 16. Development of MCTP effects on 5HT removal by the isolated, perfused lung. Rats were treated with 5 mg MCTP/kg or 0.5 ml DMF/kg, i.v. 5HT removal was determined at various times after injection as described in "Materials and Methods". Bars are mean + SEM of groups of 7-13 rats. Open bars- DMF, hatched bars-MCTP. * Significant difference from DMF group of same day (Student's t-test, $p < 0.05$).

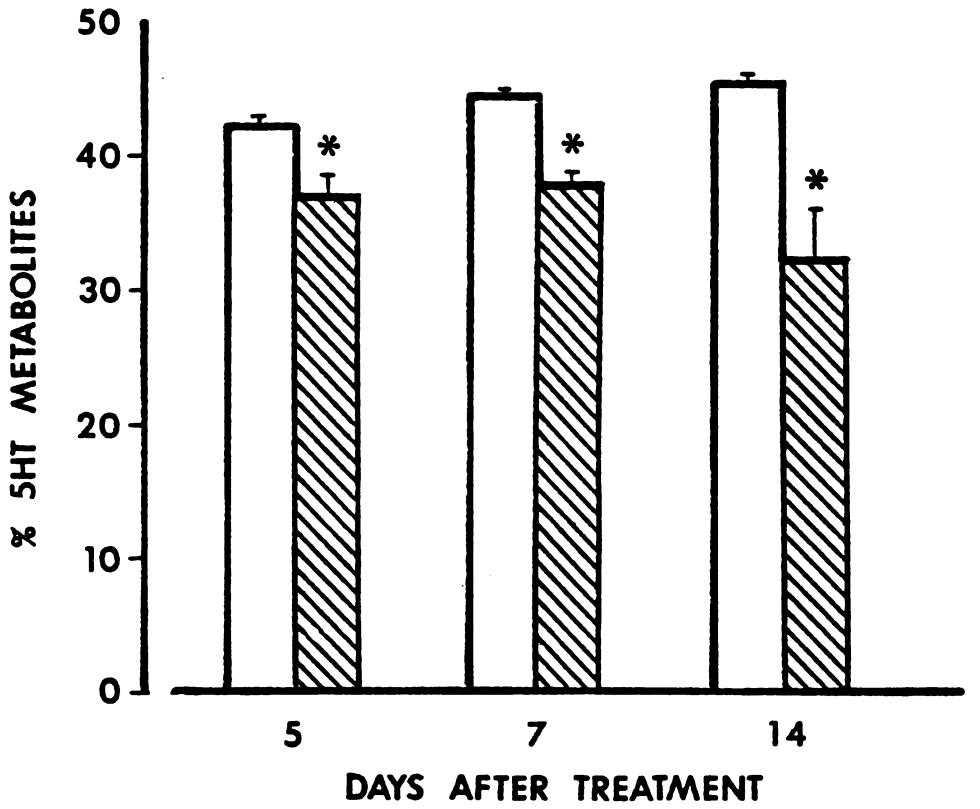


Figure 17. Development of MCTP effects on 5HT metabolite production by the isolated, perfused lung. Rats were treated with 5 mg MCTP/kg or 0.5 ml DMF/kg, i.v. The percentage of perfused 5HT in the effluent as metabolites was determined at various times after injection as described in "Materials and Methods". Bars are mean + SEM of groups of 7-13 rats. Open bars-DMF, hatched bars- MCTP. * Significant difference from DMF group of same day (Student's t-test, $p < 0.05$).

Figure 18. Comparison of the effects of MCT and MCTP on platelet counts. Platelet counts were determined in venous blood obtained during surgical procedures or in blood obtained by cardiac puncture at various times after treatment. Points represent means of 3-9 rats. SEM is not shown in order to simplify the figure. Control treatments are indicated by dashed lines (saline ▲, DMF ●). MCT (Δ) and MCTP (○) treatments are indicated by solid lines.

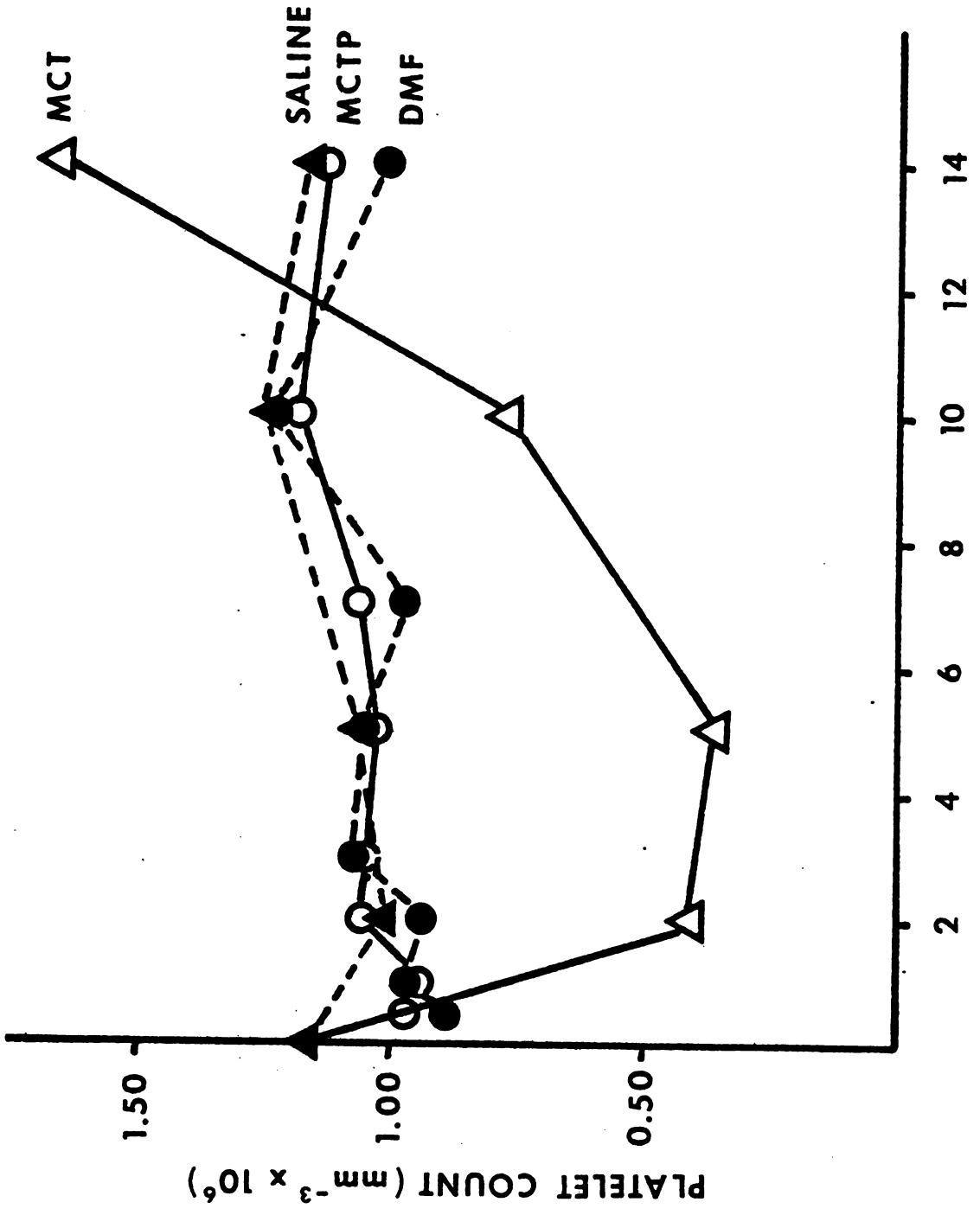

MCT
SALINE
MCTP
DMF
1.50
1.00
0.50
PLATELET COUNT ($mm^{-3} \times 10^6$)
2
4
6
8
10
12
14
TIME (DAYS)

Figure 18.

Initial experiments established the time course and concentration dependence of 5HT uptake by lung slices. Slices were incubated in buffer containing 1 μM ^{14}C-5HT for times ranging from zero to fifteen minutes, with or without 500 μM imipramine added to the medium. The 5HT uptake was linear for at least fifteen minutes. The presence of imipramine in the medium decreased 5HT uptake by about 80%. Five minutes was selected as the standard incubation time for subsequent 5HT uptake experiments.

Concentration dependence of 5HT uptake was determined over the range of 0.1-100 μM 5HT in the medium. Uptake increased linearly with concentration at concentrations greater than 10 μM, but below this concentration uptake was non-linear, suggesting that 5HT was taken into lung by both carrier-mediated and passive processes. Subtraction of the linear component from the total uptake values at low concentrations yielded a Michaelis-Menten curve from which a Lineweaver-Burk transformation was used to estimate the K_m and V_{max} for uptake. These were calculated to be 3.9 μM and 1.8 pmol/mg-min, respectively, values which are in agreement with those reported by others (Smith, L.L. et al., 1976; Wikberg and Hede, 1981). A 1 μM 5HT concentration was selected as the standard concentration for further 5HT uptake studies.

To determine whether MCT-induced lung injury causes a decrease in the uptake of 5HT by lung slices as it does in the IPL, rats were treated with MCT by three different regimens, all known to decrease 5HT removal in the IPL. Figure 19 shows the results of feeding rats 22 μg MCT/ml in their drinking water for three weeks. It had previously been shown that 5HT removal by the IPL was decreased 37% from

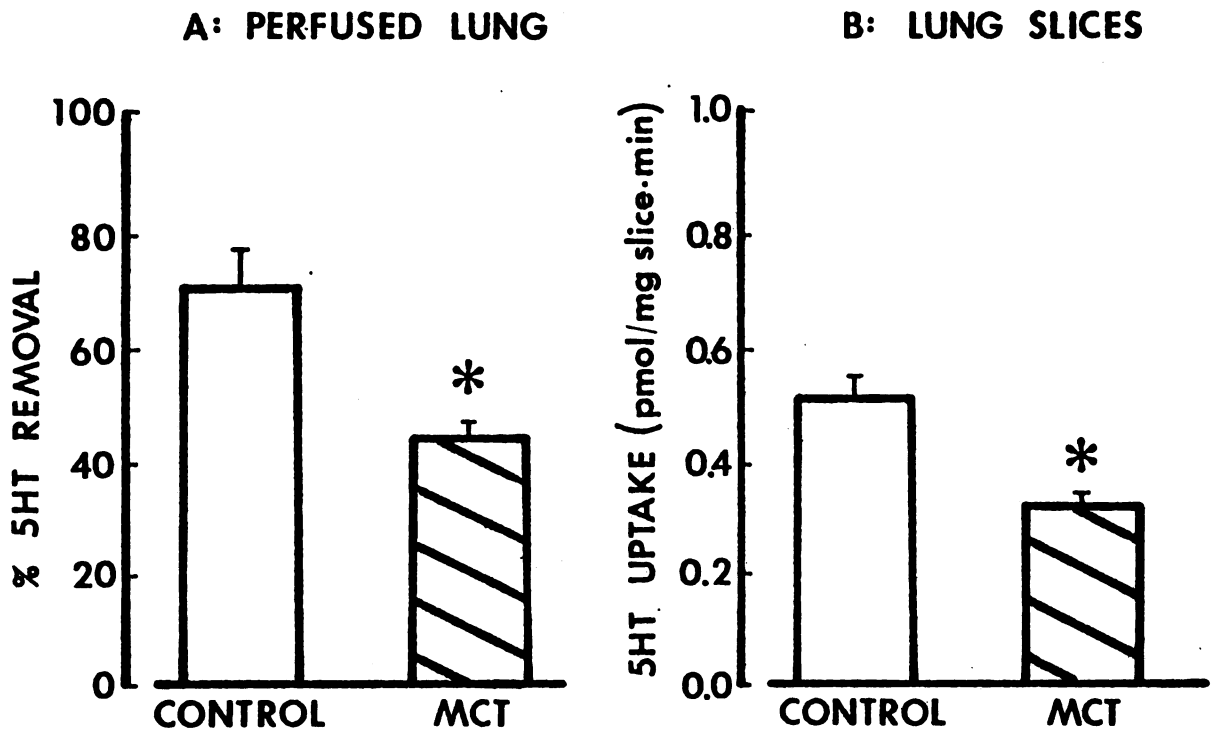


Figure 19. Comparison of 5HT uptake by the isolated, perfused lung and lung slices after MCT ingestion. Rats were fed 22 µg MCT/ml in their drinking water for 21 days. Controls drank tap water. Values for 5HT removal by the perfused lung come from Roth et al., 1981. 5HT uptake by lung slices was evaluated as described in "Materials and Methods". Bars are mean ± SEM. N=6 for each group. * Significant difference from corresponding control group (Student's t-test, $p < 0.05$).

control (Roth et al., 1981). 5HT uptake by slices in our experiments was decreased 39% from control. Rats given a single injection of MCT (60 mg/kg, s.c.) 9 days earlier had a 21% decrease in 5HT removal by the IPL and a 21% decrease in 5HT uptake by lung slices (Figure 20). Fourteen days after MCTP administration, 5HT removal by the IPL was decreased 23% and 5HT uptake by slices was decreased 35% (Figure 21). Based on these studies, the uptake of 5HT seemed to be decreased to about the same extent in the IPL and slice systems after treatment in vivo.

To examine effects of exposure of lung slices to MCT metabolites in vitro, lung slices from untreated rats were incubated for 5 hours with a pyrrole generating system consisting of liver slices and MCT. At the end of the incubation period, the slices were rinsed with fresh KRB. Preliminary experiments indicated that the generating system produced substantial amounts of pyrrole (Table 9). Incubation of MCT with lung slices alone resulted in little pyrrole accumulation. However, lung slices incubated with the generating system contained a considerable amount of pyrrole by the end of the incubation period.

5HT accumulation was measured in lung slices preincubated with the pyrrole generating system. As a control, lung slices were incubated with liver slices but without MCT for the same period. After the preincubation, the lung slices were rinsed and removed to fresh KRB containing iproniazid, and 5HT uptake was measured. Five hours of preincubation did not alter 5HT accumulation by the lung slices or the degree to which imipramine inhibited the uptake. The uptake of 5HT by

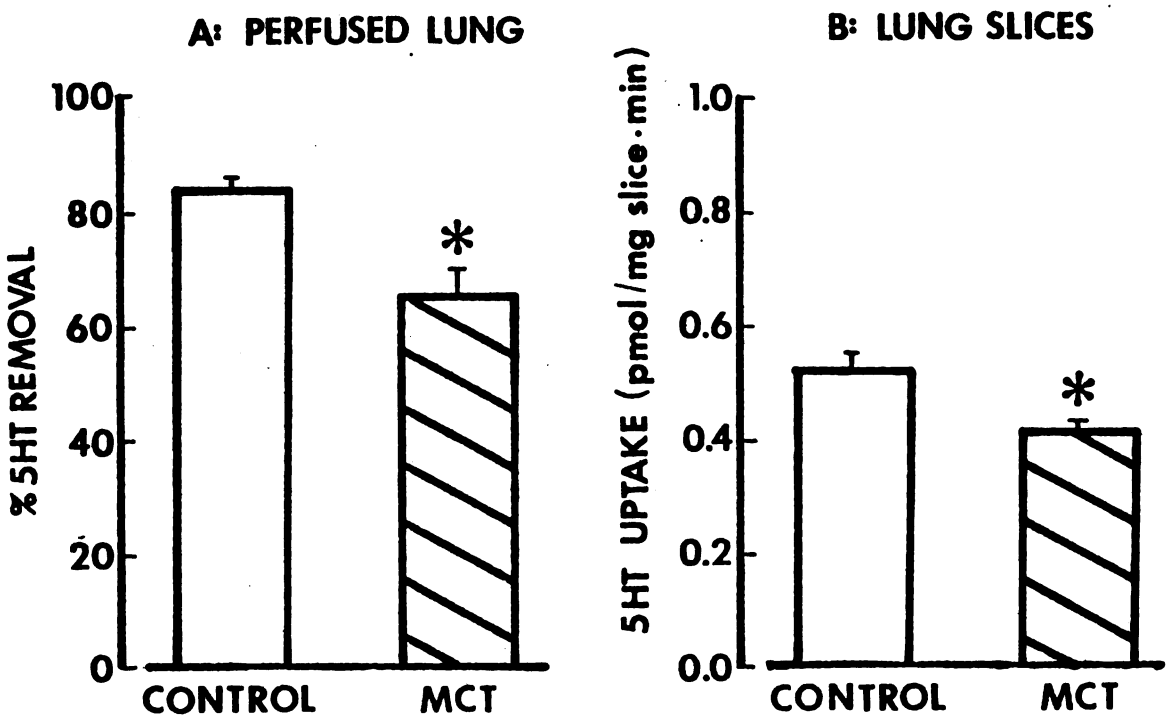


Figure 20. Comparison of 5HT uptake by the isolated, perfused lung and lung slices after a single injection of MCT. Rats were treated with 60 mg MCT/kg or 0.9% saline, s.c., 9 days prior to sacrifice. 5HT removal by the perfused lung and 5HT uptake by lung slices were evaluated as described in "Materials and Methods". Bars are mean + SEM of groups of 4-6 rats. * Significant difference from corresponding control group (Student's t-test, $p < 0.05$).

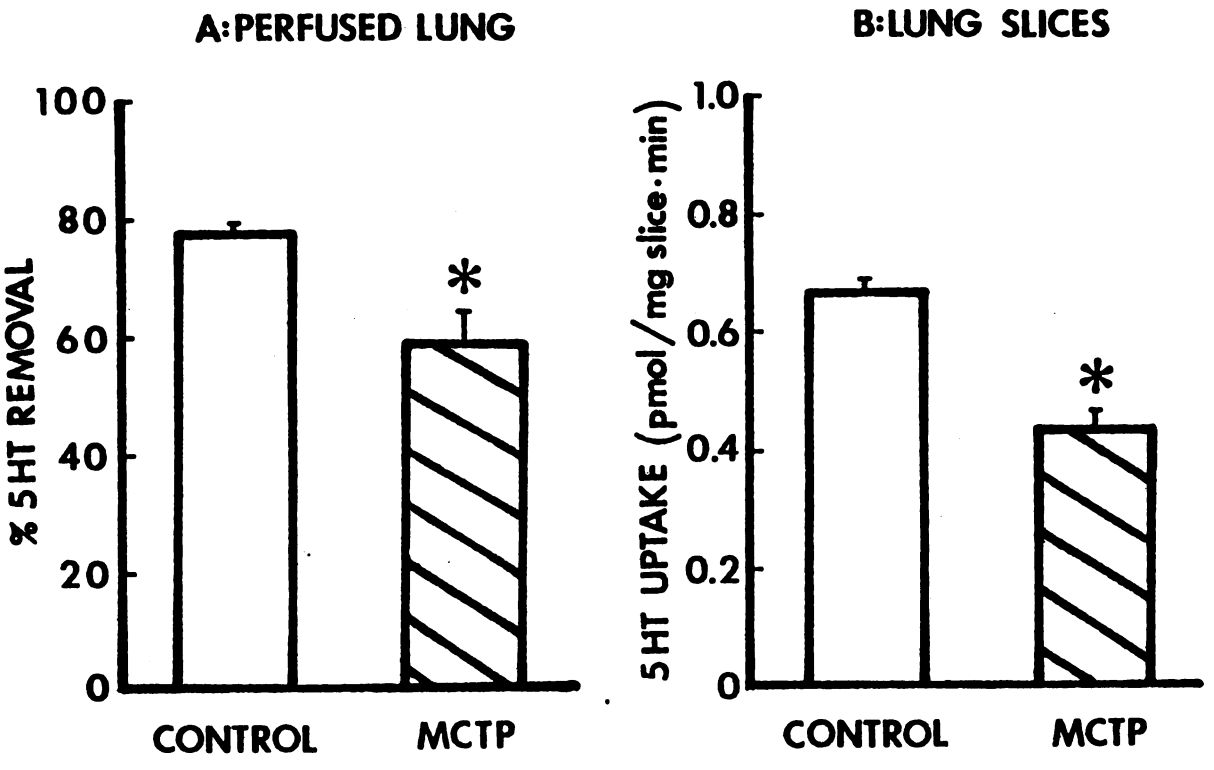


Figure 21. Comparison of 5HT uptake by the isolated, perfused lung and lung slices after a single injection of MCTP. Rats were treated with 5 mg MCTP/kg or 0.5 ml DMF/kg, i.v., 14 days prior to sacrifice. 5HT removal by the perfused lung and 5HT uptake by lung slices were evaluated as described in "Materials and Methods". Bars are mean + SEM of groups of 6 rats. * Significant difference from corresponding control group (Student's t-test, $p < 0.05$).

TABLE 9

Pyrrole Concentration in Lung Slices Exposed to a Pyrrole Generating System[a]

Incubation System	Lung Slices Concentration (µg pyrrole/g tissue)	Medium Concentration (µg pyrrole/3 ml medium)
Lung Slices, 1.5 mg MCT	4±2	1±0
Lung Slices, liver Slices, 1.5 mg MCT	51±2	101±6

[a]Slices of lung or phenobarbital-induced liver were prepared as described in "Materials and Methods" and incubated for 5 hours in 3 ml KRB under 95% O_2/5% CO_2 at 37°C. Pyrrole was estimated using a modified Ehrlich reaction with MCTP as a standard. Values reported are mean ± S.E.M. N = 3.

lung slices was the same whether they had been preincubated in the presence or absence of MCT and metabolites (Table 10).

Lung slices were also exposed to chemically synthesized MCTP. Slices were incubated for 5 hours with concentrations of 0.1-1.0 mM MCTP or appropriate volumes of the vehicle, DMF. The volumes of DMF used did not affect 5HT uptake. After 5 hours the lung slices were transferred to fresh KRB for the determination of 5HT uptake. There was no significant difference in the 5HT uptake between the treated and control groups at any MCT concentration (Table 11).

Paraquat is also accumulated in lung tissue. Like 5HT, PQ uptake is primarily carrier-mediated, with a minor passive component (Lock et al., 1976; Rose et al., 1976). Unlike 5HT, PQ accumulates in the lung without being metabolized. Histochemical studies indicate that the site of PQ uptake is the alveolar Type II cell and not the endothelium (Waddell and Marlowe, 1980). This is supported by experiments showing that perfused PQ is taken up very slowly into isolated lung, where an endothelial barrier must be crossed, but accumulates rapidly into slices of lung, where PQ has direct access to alveolar epithelium (Orton et al., 1973). In addition, PQ and 5HT are apparently taken up by different transport processes since these compounds do not effectively inhibit the transport of one another into lung slices (Smith, L.L. et al., 1976). Since MCT has been shown to cause some changes in alveolar epithelial cells (Valdivia et al., 1967), it was of interest to examine PQ uptake in lungs treated in vivo or in vitro with MCTP.

The time course of PQ uptake was determined by incubating lung slices for 0-30 minutes at 1, 10 and 100 μM concentrations of PQ. At

TABLE 10

Effect of Incubation with a Pyrrole Generating System on 5HT Uptake by Rat Lung Slices

System Components[a]	5HT Uptake by Lung Slice[b] (pmol/mg slice-minute)
Lung Slices, Liver Slices No MCT	0.61±0.05
Lung Slices, Liver Slices 930 μg MCT/ml	0.58±0.05

[a]Lung slices were preincubated for 5 hours at 37°C with the pyrrole generating systems. After transfer to fresh incubation medium, 5HT uptake by the lung slices was determined as described in "Materials and Methods". Results are expressed as mean ± S.E.M. N = 4.

[b]Data were analyzed using Student's t-test. There was no significant difference between the two groups ($p<0.05$).

TABLE 11

5HT Uptake by Rat Lung Slices Exposed to MCTP *In Vitro*

Treatment[a]	MCTP Concentration (mM)[b]			
	0.10	0.25	0.50	1.00
Control	0.79±0.01	0.74±0.00	0.81±0.03	0.62±0.01
MCTP	0.72±0.02	0.82±0.01	0.72±0.03	0.62±0.02
% of Control	91.4	112.7	89.2	98.7

[a]Lung slices were preincubated for 5 hours at 37°C with appropriate concentrations of MCTP or DMF. The slices were analyzed for 5HT uptake as described in "Materials and Methods". Results are expressed as mean ± S.E.M. in pmol/mg slice-min. N = 6. Each animal served as its own control and received all treatments.

[b]Data were analyzed by block factorial analysis of variance. There were no significant changes in 5HT uptake in the treated lung slices at any of the MCTP concentrations ($p<0.05$).

the lower concentrations, uptake increased linearly with time until at least 30 minutes; at 100 μM PQ, uptake was no longer linear by 30 minutes. Ten minutes was selected as the standard incubation time for further PQ uptake experiments, as uptake was linear with time for all concentrations.

The concentration dependence of PQ uptake with or without the uptake inhibitor spermine in the medium was established over the range of 1-1000 μM PQ. At a PQ concentration of 10 μM, 500 μM spermine decreased uptake by about 66%. Uptake of PQ increased linearly with concentration at concentrations greater than 250 μM, but below this concentration uptake was not linear. This suggests that PQ was taken into the lung slices by both carrier-mediated and passive processes. From a Lineweaver-Burk transformation of the saturable component, the K_m and V_{max} were calculated to be 106 μM and 7.3 pmol/mg-minute, respectively. These results are in agreement with the values reported by others (Ross and Krieger, 1981). A PQ concentration of 10 μM was selected for further PQ uptake studies.

Lung slices from the animals treated with 5 mg/kg MCTP or DMF vehicle i.v. were evaluated for their ability to accumulate PQ. These results are shown in Figure 22. A small but statistically significant decrease in PQ uptake was observed in lungs from treated animals, PQ uptake being diminished by about 15%.

Lung slices which had been preincubated in vitro with the various concentrations of MCTP for 5 hours were tested for their ability to accumulate PQ. Results are shown in Table 12. There was no significant difference in PQ uptake between the treated and control groups at any MCTP concentration.

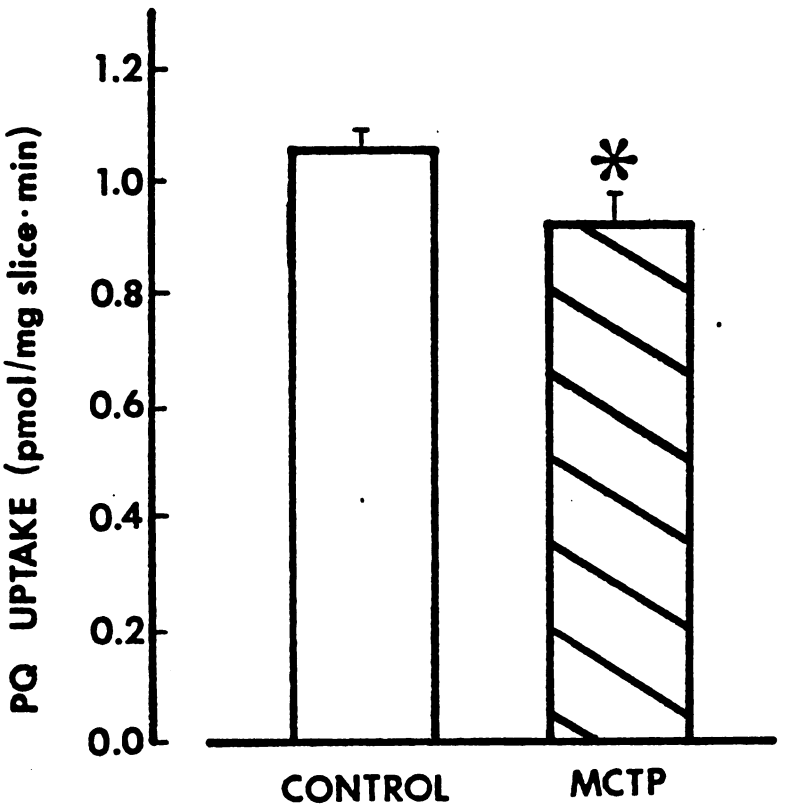


Figure 22. Effect of MCTP treatment on paraquat uptake by lung slices. Rats were treated with 5 mg MCTP/kg or 0.5 ml DMF/kg, i.v. PQ uptake was determined 14 days later as described in"Materials and Methods". Bars are mean ± SEM. N=6. * Significant difference from DMF group (Student's t-test, $p < 0.05$).

TABLE 12

Paraquat Uptake by Rat Lung Slices Exposed to MCTP
In Vitro

Treatment[a]	MCTP Concentration (mM)[b]			
	0.10	0.25	0.50	1.00
Control	0.94±0.06	0.95±0.17	0.88±0.03	0.82±0.04
MCTP	1.04±0.20	0.82±0.09	0.82±0.02	0.74±0.04
% of Control	109.9	86.3	92.2	90.1

[a]Lung slices were preincubated for 5 hours at 37°C with appropriate concentrations of MCTP or DMF. The slices were analyzed for PQ uptake as described in "Materials and Methods". Results are expressed as mean ± S.E.M. in pmol/mg slice-min. N = 4. Each animal served as its own control and received all treatments.

[b]Data were analyzed by block factorial analysis of variance. There were no significant changes in PQ uptake in the treated lung slices at any of the MCTP concentrations ($p<0.05$).

F. In Vitro Exposure of Isolated, Perfused Lungs to MCTP

In another attempt to show whether MCTP could directly damage lung tissue, isolated, perfused lungs were exposed to MCTP via the vasculature. In the first experiments, a high dose of MCTP (500 μM) was added to the recirculating reservoir and perfused through the lung for 90 minutes. This dose was chosen because it was cytotoxic to hamster V79 cells in culture (D. Doolittle, unpublished results) and rapidly causes severe edema when given to an intact animal (Plestina and Stoner, 1972). The results of this experiment are summarized in Table 13. The lack of change in perfusion pressure over the duration of the perfusion and of the wet/dry lung weight ratios indicate that no edema developed. The concentration of MCTP in the perfusion medium was consistently lower at the end of the perfusion than at the beginning but there was still a large amount of the Ehrlich reactive material at the end of the perfusion. Lung pyrrole content was not measured. After the recirculating perfusion, the reservoir was switched to one free of MCTP and 5HT removal was determined. Neither the percentage of 5HT removal nor that metabolized was altered by the 90 minute exposure.

Since 500 μM MCTP in the reservoir had no acute effects on the IPL, the next approach was to infuse the same quantity of MCTP into the pulmonary artery cannula of the IPL. The results of this manipulation are summarized in Table 14. The original plan was to perfuse in a recirculating manner for 60-90 minutes, then switch to fresh perfusion medium and measure 5HT uptake. All of the MCTP-treated lungs became severely edematous from 35 to 60 minutes after the start

TABLE 13

Effects of 8.1 mg MCTP in Reservoir on IPL[a]

	DMF	MCTP
ΔP (mmHg)	0±0	0±0
Wet/Dry Lung Weight	5.8±0.3	6.1±0.1
$[MCTP]_I$ (μM)	0	118±30
$[MCTP]_F$ (μM)	0	75±24
% 5HT Removal	70.2±2.0	75.9±1.8
% 5HT Metabolites	41.7±1.2	43.0±1.5

[a]Lungs from untreated rats were perfused in a recirculating manner as described in "Materials and Methods" for 90 minutes. The 50 ml of recirculating medium contained either 0.5 ml DMF or 8.1 mg MCTP in 0.5 ml DMF. "ΔP" is the difference between the initial perfusion pressure when the MCTP or DMF was added to the reservoir and the end of the 5HT perfusion. $[MCTP]_I$ and $[MCTP]_F$ are the concentrations in the recirculating perfusion medium at the beginning and the end of the perfusion. After 90 min of perfusion, the reservoir was switched to one containing ^{14}C-5HT, and lungs perfused in a single pass manner to determine 5HT removal. Values reported are mean ± S.E.M. N = 4. There were no statistically significant differences between the groups (Student's t-test, $p<0.05$).

TABLE 14

Effects of 8.1 mg MCTP via PA Cannula on IPL[a]

	DMF	MCTP
ΔP (mmHg)	0±0	5±1[b]
Wet/Dry Lung Weight	6.2±1.0	38.2±5.0[b]
$[MCTP]_I$ (μM)	0	20±2
$[MCTP]_F$ (μM)	0	18±2
Lavage Fluid LDH (U/100 ml)	8.0±1.4	4.5±0.6[b]
Edema Fluid LDH (U/100 ml)	---	14.6±1.1
Total LDH (U)	0.74(0.51-1.41)	1.30(0.70-2.04)
Lavage Fluid Protein	0.63±0.23	2.48±0.15[b]

[a]Lungs from untreated rats were perfused in a recirculating manner as described in "Materials and Methods" for 30-60 minutes. After equilibration, 8.1 mg MCTP dissolved in 0.2 ml DMF or 0.2 ml DMF alone was infused into the pulmonary artery (PA) cannula over a 4 minute period. "ΔP" is the difference between the perfusion pressure at the start of MCTP or DMF infusion and the end of the perfusion. $[MCTP]_I$ was determined 5 minutes after the start of MCTP infusion. $[MCTP]_F$ was the concentration in the perfusion medium at the end of the perfusion. LDH refers to lactate dehydrogenase activity in the designated cell-free fluid. Values reported are mean ± S.E.M. except for total LDH which is mean (range). N = 6.

[b]Significantly different from DMF group (Student's t-test, $p<0.05$).

of MCTP infusion precluding measurement of 5HT uptake. The change in perfusion pressure and the increase in wet/dry lung weight ratios confirm the visual evidence of edema in the MCTP-treated lungs. The rise in perfusion pressure was fairly rapid but levelled off abruptly, suggesting that a mechanical constraint limited the maximum pressure attainable in this particular system. The rise in pressure did not start until 30-40 minutes after MCTP infusion. There was no change in perfusion pressure while the MCTP or DMF were being infused. The concentration of MCTP in the perfusion medium was much lower in this experiment than the previous one despite the fact that the same amount of MCTP was added to both systems. This suggests that more was being retained by the lung when it was infused directly into the PA cannula. Since the lungs were so grossly edematous, 5HT uptake was not performed. To provide some quantitative indication of lung injury, the release of lactate dehydrogenase (LDH) activity into the perfusate and the airways was measured. Figure 23 shows the LDH activity in the perfusate as a function of time after MCTP/DMF infusion. Perfusate LDH activity was increased in the treated lungs at the end of the perfusion period but was not different from DMF-infused lungs at earlier times. LDH activity in the cell-free lavage fluid of MCTP-treated lungs was actually lower than that of the DMF lungs (Table 14). As might be expected, there was considerable LDH activity in the edema fluid removed from the treated lungs before lavage. The total LDH activity released into the airways was estimated by calculating the amount of LDH activity in the lavage fluid volume recovered and the edema fluid, then adding these together. Although not significantly different, there was a trend towards increased total LDH

Figure 23. Appearance of lactate dehydrogenase (LDH) activity in the perfusate after exposure of IPL to MCTP *in vitro*. Lungs were perfused in a recirculating manner as described in "Materials and Methods." At various times after the infusion of 25 μmoles MCTP or 0.2 ml DMF into the pulmonary artery cannula, aliquots of perfusion medium were removed from the reservoir for measurement of LDH activity. Points represent mean + SEM of groups of 6 rats. *Significant difference from DMF group at same time point (Student's t-test, $p<0.05$).

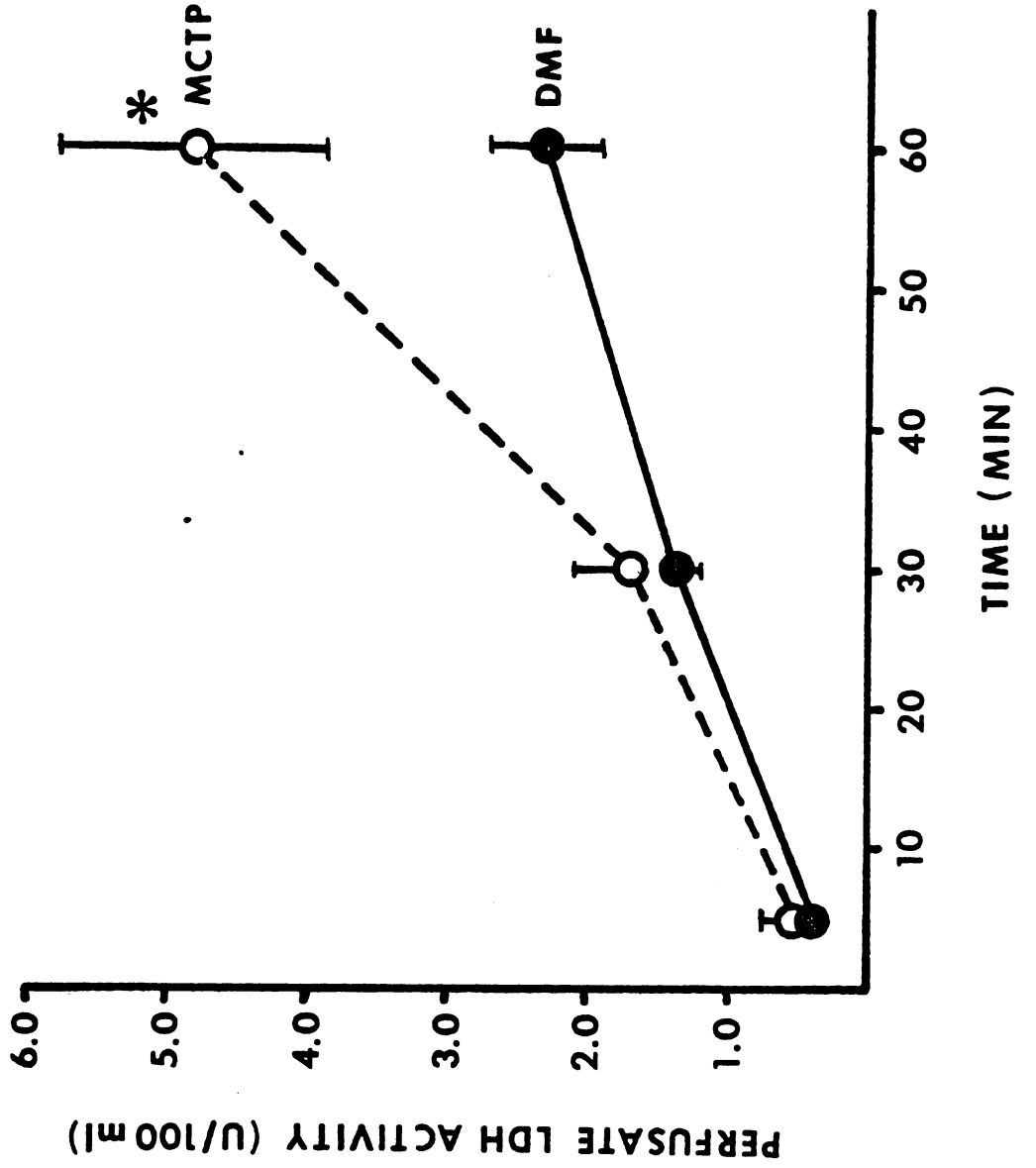

PERFUSATE LDH ACTIVITY (U/100 ml)
6.0
5.0
4.0
3.0
2.0
1.0
10
20
30
40
50
60
TIME (MIN)
MCTP
DMF
*

Figure 23.

activity in the MCTP-treated lungs. The protein concentration in the lavage fluid from MCTP-treated lungs was significantly greater than that in DMF-treated lungs.

The dose of MCTP used in this series of perfusions was extremely high (equivalent to 32 mg/kg for an intact animal). This raised the question of whether the effects after such a large dose are the same as the initial effects after a dose considerably smaller, but sufficient to cause pulmonary hypertension. To answer this query, an infusion of MCTP identical to that given to a whole animal for treatment in vivo was infused into the IPL. Specifically, each lung received 0.12 ml DMF or 1.2 mg MCTP in 0.12 ml DMF over a period of 4-5 minutes. The major difference from in vivo treatment was the speed of infusion. In vivo, the dose and flush are injected over a much shorter interval, about 30 seconds. The initial plan was to do a recirculating perfusion for 60 minutes, then to switch the perfusion medium and determine 5HT removal. The treated lungs started becoming edematous 30-45 minutes after infusion of MCTP, however, so all subsequent recirculating perfusions were stopped after 30 minutes to allow reliable estimation of 5HT removal.

The results are summarized in Table 15. MCTP treatment caused an increase in the perfusion pressure over the course of the perfusion and an increase in the wet/dry lung weight ratio, both indicative of edema. There were no changes in perfusion pressure during the DMF or MCTP infusions. 5HT removal was not altered by MCTP-infusion but the amount in the effluent as metabolite was decreased by 11%. Lavage fluid LDH activity was not altered, but the MCTP-exposed lungs had

TABLE 15

Effects of 1.2 mg MCTP via PA Cannula on IPL[a]

	DMF	MCTP
ΔP (mmHg)	1±0	7±2[b]
Wet/Dry Lung Weight	5.2±0.3	31.5±7[b]
% 5HT Removal	75.7±1.0	75.0±3.6
% 5HT Metabolite	51.0±1.4	45.3±1.1[b]
Lavage Fluid LDH (U/100 ml)	5.9±1.1	4.5±2.1
Edema Fluid LDH (U/100 ml)	---	10.7±5.3
Total LDH (U)	0.67(0.44-1.06)	0.76(0.41-1.57)

[a]Lungs from untreated rats were perfused in a recirculating manner as described in "Materials and Methods" for 30 minutes. After equilibration, 1.2 mg MCTP dissolved in 0.12 ml DMF or 0.12 ml DMF alone was infused into the pulmonary artery (PA) cannula over a 4-8 minute period. "ΔP" is the difference between the perfusion pressure at the start of MCTP or DMF infusion and the end of the perfusion. $[MCTP]_I$ was determined 5 minutes after the start of MCTP infusion. $[MCTP]_F$ was the concentration in the perfusion medium at the end of the recirculating perfusion. The reservoir was switched to one containing ^{14}C-5HT after 30 minutes and a single pass perfusion to determine 5HT removal was performed. LDH refers to lactate dehydrogenase activity in the designated cell-free fluid. Values reported are mean ± S.E.M. except for total LDH which is mean (range). N = 5.

[b]Significantly different from DMF group (Student's t-test, $p<0.05$).

edema fluid in the airways which contained LDH activity. The estimation of total LDH activity showed no differences between the DMF and MCTP-treated lungs. Measurement of LDH activity in the recirculating perfusion medium also showed no difference between lungs from DMF- and MCTP-treated rats.

G. Effects of Thrombocytopenia on MCTP Toxicity

To ascertain whether platelets were involved in MCTP-induced cardiopulmonary changes, the effects of thrombocytopenia (TCP) on MCTP toxicity were examined. Thrombocytopenia of 48-72 hour duration was induced by administration of goat anti-rat platelet antiserum. Preliminary experiments indicated that a dosage regimen of two 0.5 ml injections (i.p.) of PAS was optimal with respect to duration and magnitude of the decrease in platelet count. Attempts to increase the duration or magnitude of the effect had adverse effects on the health of the animals (decreased hematocrit, loss of body weight, death). The effects of this dosage regimen of PAS on the circulating platelet count are shown in Figure 24. Platelet counts were reduced to 10% of control for 12-24 hours after the first injection of PAS and were less than 20% of control values until 48 hours after the first injection. Four days after PAS, the platelet counts were still reduced but to a much smaller extent. Treatment in the same manner with CS did not alter the circulating platelet count.

Three sets of experiments were done, differing only in when the antiserum was administered relative to the MCTP. In the first set, antiserum was administered 12 hours before MCTP so that the platelet

Figure 24. Effects of PAS on platelet count. Rats were given 2 doses of 0.5 ml PAS (i.p.), 18 hours apart (indicated by arrows under abscissa). Platelets were counted in citrated blood obtained by cardiac puncture at various times after PAS administration. Each rat was bled only once. Points are mean $\pm$ SEM. Point at time zero based on 41 rats, all other points determined in groups of 4-6 rats.

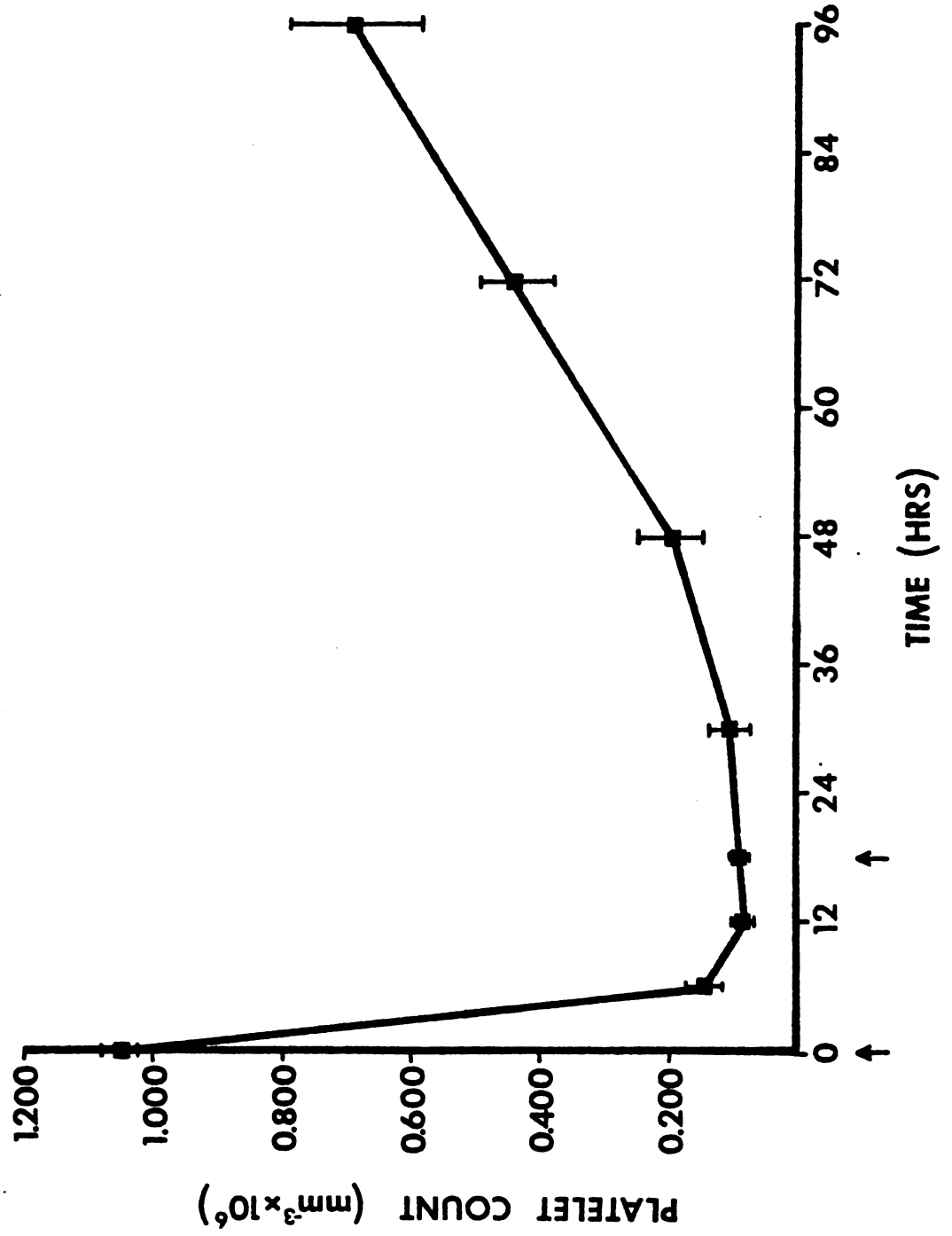

PLATELET COUNT (mm³×10⁶)
1.200
1.000
0.800
0.600
0.400
0.200
0
12
24
36
48
60
72
84
96
TIME (HRS)

Figure 24.

count would be decreased for days 0-2 after MCTP treatment. Antiserum was administered 3 days after MCTP in the second set of experiments, resulting in thrombocytopenia for days 3-5 after MCTP treatment. The third set of experiments was done with the rats thrombocytopenic for days 6-8 after MCTP treatment. Animals were sacrificed 14 days after MCTP administration in all experiments and lung damage and pulmonary hypertension were evaluated.

These three periods were chosen for thrombocytopenia induction because of their potential to provide information about the mechanism by which platelets were involved in the generation of PH. By administering MCTP to thrombocytopenic rats (0-2 day period), information about whether platelets are involved in the initiation of injury was expected. The other two time periods bracket significant events in the development of MCTP effects. The first evidence of functional lung injury was observed 4 days after MCTP administration and increased pulmonary arterial pressure was first detectable 7 days after MCTP administration (Bruner et al., 1983).

Treatment with MCTP caused a decreased final body weight and reduced the body weight gain over the treatment period (Table 16). Reduction of the platelet count at any of the time intervals tested did not affect final body weight or weight gain in the DMF-treated rats. In addition, thrombocytopenia did not alter the effects of MCTP. Hematocrit, platelet and white blood cell counts were not different in any of the treatment groups.

The ability of the isolated lung to remove 5HT from the perfusion medium was decreased approximately 16% in MCTP- compared to DMF-treated

TABLE 16

Effects of MCTP and PAS on Body Weight

Parameter	Period of Thrombocytopenia	Treatment[a]			
		CS/DMF	PAS/DMF	CS/MCTP	PAS/MCTP
Final Body Weight (g)	0-2 Days	329±5 (12)	339± 7 (11)	297±11[b] (12)	301± 9[c] (12)
	3-5	329±5 (12)	331± 9 (12)	259±14[b] (8)	271±18[c] (9)
	6-8	346±9 (12)	336±11 (12)	276±18[b] (11)	271±19[c] (10)
Body Weight Gain (g)	0-2 Days	87±9 (12)	101± 5 (12)	74± 8[d] (12)	71±10[e] (12)
	3-5	92±3 (12)	90± 4 (12)	14±15[d] (8)	27±16[e] (9)
	6-8	103±5 (12)	91± 6 (12)	25±16[d] (11)	31±18[e] (10)

[a]As described in "Materials and Methods", rats were treated with 5 mg MCTP/kg or 0.5 ml DMF/kg on day zero and control serum (CS) or anti-platelet serum (PAS) on the days indicated. Fourteen days after MCTP treatment, the animals were weighed. Body weight gain is the difference between the final body weight at 14 days and the weight at the time of MCTP dosing. Values represent mean ± S.E.M. The number in each group is in parentheses.

[b]Statistically significant difference from CS/DMF group (2-way factorial ANOVA, Tukey's ω-procedure, $p<0.05$).

[c]Statistically significant difference from PAS/DMF group (2-way factorial ANOVA, Tukey's ω-procedure, $p<0.05$).

[d]Statistically significant difference from CS/DMF group (Rank sum test, $p<0.05$).

[e]Statistically significant difference from PAS/DMF group (Rank sum test, $p<0.05$).

rats (Table 17). The percentage of 5HT converted to metabolites was also decreased in the MCTP-treated lungs compared to DMF lungs. In addition, the baseline perfusion pressure was elevated in the MCTP-treated lungs. Thrombocytopenia alone did not alter any of these parameters, nor did it alter the magnitude of the response to MCTP.

Wet lung weights were increased by MCTP treatment but not by PAS at any of the periods tested. Wet/dry lung weights were increased in some groups treated with MCTP, but not all of them (Table 18). Dry lung weights were elevated in all MCTP-treated groups except the group made thrombocytopenic for days 6-8 (data not shown).

Analysis of the bronchopulmonary lavage fluid indicated that MCTP treatment increased lavage fluid LDH activity and protein concentration (Table 19). PAS had no effect of its own at any of the time periods examined, nor did it alter the effect of MCTP. Plasma LDH activity was unchanged by MCTP or PAS treatment (data not shown).

Right ventricular hypertrophy, evidenced by increases in the ratio of RV/(LV+S), were observed in the MCTP-treated groups (Figure 25). Induction of thrombocytopenia for days 3-5 or days 6-8 of the MCTP treatment period reduced the development of right heart hypertrophy by 19 or 41%, respectively. The groups of rats treated with PAS and MCTP still had significant hypertrophy compared to DMF rats but the magnitude was reduced considerably. The ratio (LV+S)/BW was not different in any of the treatment groups.

TABLE 17

Effects of MCTP and PAS on Isolated Perfused Lung Parameters

Parameter	Period of Thrombocytopenia	Treatment[a]			
		CS/DMF	PAS/DMF	CS/MCTP	PAS/MCTP
Initial Perfusion Pressure (mmHg)	0-2 Days	7±0 (11)	6±0 (11)	11±0[b] (12)	10±0[c] (12)
	3-5	6±0 (11)	6±0 (12)	10±0[b] (8)	9±0[c] (9)
	6-8	6±0 (10)	6±0 (11)	9±1[b] (10)	9±1[c] (10)
% 5HT Removal	0-2 Days	73.5±1.1 (11)	78.6±2.0 (11)	60.0±2.5[b] (12)	65.7±2.9[c] (12)
	3-5	76.2±1.5 (13)	75.3±1.1 (13)	65.0±3.2[b] (9)	63.8±4.7[c] (9)
	6-8	76.8±2.3 (10)	79.1±1.5 (11)	64.4±3.5[b] (10)	66.3±4.7[c] (10)
% 5HT Metabolites	0-2 Days	47.3±4.2 (11)	51.3±2.2 (11)	41.0±2.1[b] (12)	42.2±3.1[c] (12)
	3-5	48.0±1.1 (13)	46.8±0.9 (13)	35.3±1.9[b] (9)	35.9±2.8[c] (9)
	6-8	49.7±2.0 (10)	50.2±1.2 (11)	38.1±2.4[b] (10)	41.1±3.4[c] (10)

[a]As described in "Materials and Methods", rats were treated with 5 mg MCTP/kg or 0.5 ml DMF/kg on day zero and control serum (CS) or anti-platelet serum (PAS) at the times indicated. Fourteen days after MCTP treatment, the lungs were perfused to determine 5HT uptake and metabolism ability. Values represent mean ± S.E.M. The number in each group is in parentheses.

[b]Statistically significant difference from CS/DMF group (2-way factorial ANOVA, Tukey's ω-procedure, $p<0.05$).

[c]Statistically significant difference from PAS/DMF group (2-way factorial ANOVA, Tukey's ω-procedure, $p<0.05$).

TABLE 18

Effects of MCTP and PAS on Lung Weight

Parameter	Period of Thrombocytopenia	Treatment[a]			
		CS/DMF	PAS/DMF	CS/MCTP	PAS/MCTP
Wet Lung Wt (g)	0-2 Days	1.532±0.073 (10)	2.044±0.255 (11)	2.989±0.218[b] (12)	4.144±0.589[c] (12)
	3-5	1.224±0.082 (12)	1.535±0.194 (12)	4.791±1.112[b] (8)	3.216±0.576[c] (19)
	6-8	1.514±0.118 (12)	1.452±0.098 (12)	4.024±0.402[b] (12)	3.691±1.205[c] (10)
Wet/Dry Lung Weight	0-2 Days	5.8±0.3 (10)	7.1±0.7 (11)	6.8±0.4[b] (11)	9.4±1.4[c] (12)
	3-5	5.1±0.3 (12)	6.2±0.8 (12)	12.7±2.7[b] (8)	9.2±1.6[c] (9)
	6-8	6.2±0.4 (12)	6.0±0.6 (12)	10.9±1.1[b] (11)	10.3±2.5 (10)

[a]Rats were treated with 5 mg MCTP/kg or 0.5 ml DMF/kg on day zero and control serum (CS) or anti-platelet serum (PAS) on the days indicated as described in "Materials and Methods". Fourteen days after MCTP treatment, the lungs were removed and perfused to determine 5HT uptake. Wet lung weights were determined after perfusion. Values represent mean ± S.E.M. The number in each group is in parentheses.

[b]Significant difference from CS/DMF group (Rank sum test, $p<0.05$).

[c]Significant difference from PAS/DMF group (Rank sum test, $p<0.05$).

TABLE 19

Effects of MCTP and PAS on Bronchopulmonary Lavage Parameters

Parameter	Period of Thrombocytopenia	Treatment[a]			
		CS/DMF	PAS/DMF	CS/MCTP	PAS/MCTP
Lavage Fluid LDH Activity (U/100 ml)	0-2 Days	7.4±2.1 (12)	6.5±1.1 (11)	12.7±2.2[b] (12)	18.9±3.5[c] (12)
	3-5	4.7±0.9 (12)	4.9±1.4 (12)	23.5±4.1[b] (9)	18.3±3.4[c] (9)
	6-8	5.3±0.6 (10)	4.2±0.4 (11)	21.2±3.2[b] (10)	17.3±4.4[c] (10)
Lavage Fluid Protein (mg/ml)	0-2 Days	0.24±0.06 (10)	0.65±0.22 (11)	1.72±0.25[b] (12)	2.02±0.31[c] (12)
	3-5	0.40±0.12 (14)	0.53±0.29 (13)	7.03±1.64[b] (9)	3.72±0.90[c] (9)
	6-8	0.60±0.17 (10)	0.24±0.04 (11)	3.66±0.77[b] (10)	3.32±1.19[c] (10)

[a]Rats were treated with 5 mg MCTP/kg or 0.5 ml DMF/kg on day zero and control serum (CS) or anti-platelet serum (PAS) on the days indicated as described in "Materials and Methods". Fourteen days after MCTP treatment, the lungs were lavaged with 0.9% saline and the lactate dehydrogenase (LDH) activity and protein concentration in the cell-free lavage fluid were measured. Values represent mean ± S.E.M. The number in each group is in parentheses.

[b]Statistically significant difference from CS/DMF group for same period of thrombocytopenia (2-way factorial ANOVA, Tukey's ω-procedure, $p<0.05$).

[c]Statistically significant difference from PAS/DMF group for same period of thrombocytopenia (2-way factorial ANOVA, Tukey's ω-procedure, $p<0.05$).

Figure 25. Effect of thrombocytopenia on MCTP-induced right ventricular hypertrophy. Rats were treated with 5 mg MCTP/kg or 0.5 ml DMF/kg, i.v., on day zero. In addition, the rats received CS or PAS at appropriate times to induce thrombocytopenia at the designated periods. RV/(LV+S) was determined 14 days after MCTP injection. Bars are mean $\pm$ SEM of groups of 8-12 rats. a = significantly different from CS/DMF group; b = significantly different from PAS/DMF group; c = significantly different from CS/MCTP group (2-way factorial ANOVA, Tukey's ω-procedure for unconfounded comparisons, $p<0.05$).

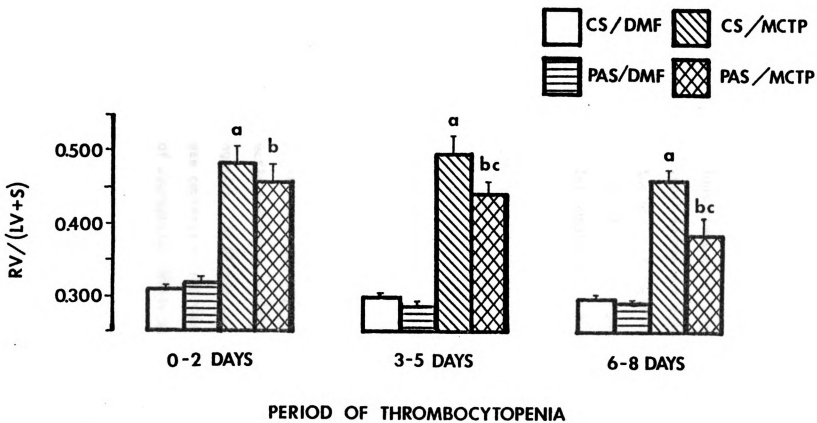

CS/DMF
CS/MCTP
PAS/DMF
PAS/MCTP
RV/(LV+S)
0.500
0.400
0.300
a
b
a
bc
a
bc
0-2 DAYS
3-5 DAYS
6-8 DAYS
PERIOD OF THROMBOCYTOPENIA

Figure 25.

H. Platelet Aggregation After MCTP Treatment In Vivo

A basis for the involvement of platelets in MCTP-induced PH may lie in changes in their response to aggregating agents. To examine this possibility, platelet-rich plasma was harvested from DMF- or MCTP-treated rats at various times after treatment and platelet aggregation in response to several agents was determined. The time periods examined were chosen on the basis of their significance in the time course of MCTP toxicity. At 24 hours, no pulmonary changes are measurable but the platelet population in the blood has been directly exposed to MCTP and there may still be some MCTP circulating. Four days after MCTP administration was the time when the first lung injury was detectable by biochemical means (Bruner et al., 1983) and at seven days the pulmonary arterial pressure was elevated. By 14 days, right ventricular hypertrophy had developed.

Aggregation responses to three agents (adenosine diphosphate (ADP), dog collagen and arachidonic acid) were evaluated. Figure 26 shows typical aggregation curves from a DMF-treated (control) rat after ADP stimulation. At low concentrations ADP stimulates platelet surface receptors directly and causes a shape change which precedes a small reversible aggregation. At higher concentrations, ADP causes the platelets to release contents of the dense granules as well, enhancing the extent of aggregation (Mills and Macfarlane, 1976). Collagen induces platelet aggregation by a different mechanism than ADP. Platelets adhere to the collagen particles and this stimulates granule release (Holmsen et al., 1969) and prostaglandin synthesis (Smith et al., 1973). Arachidonic acid is converted by platelets to

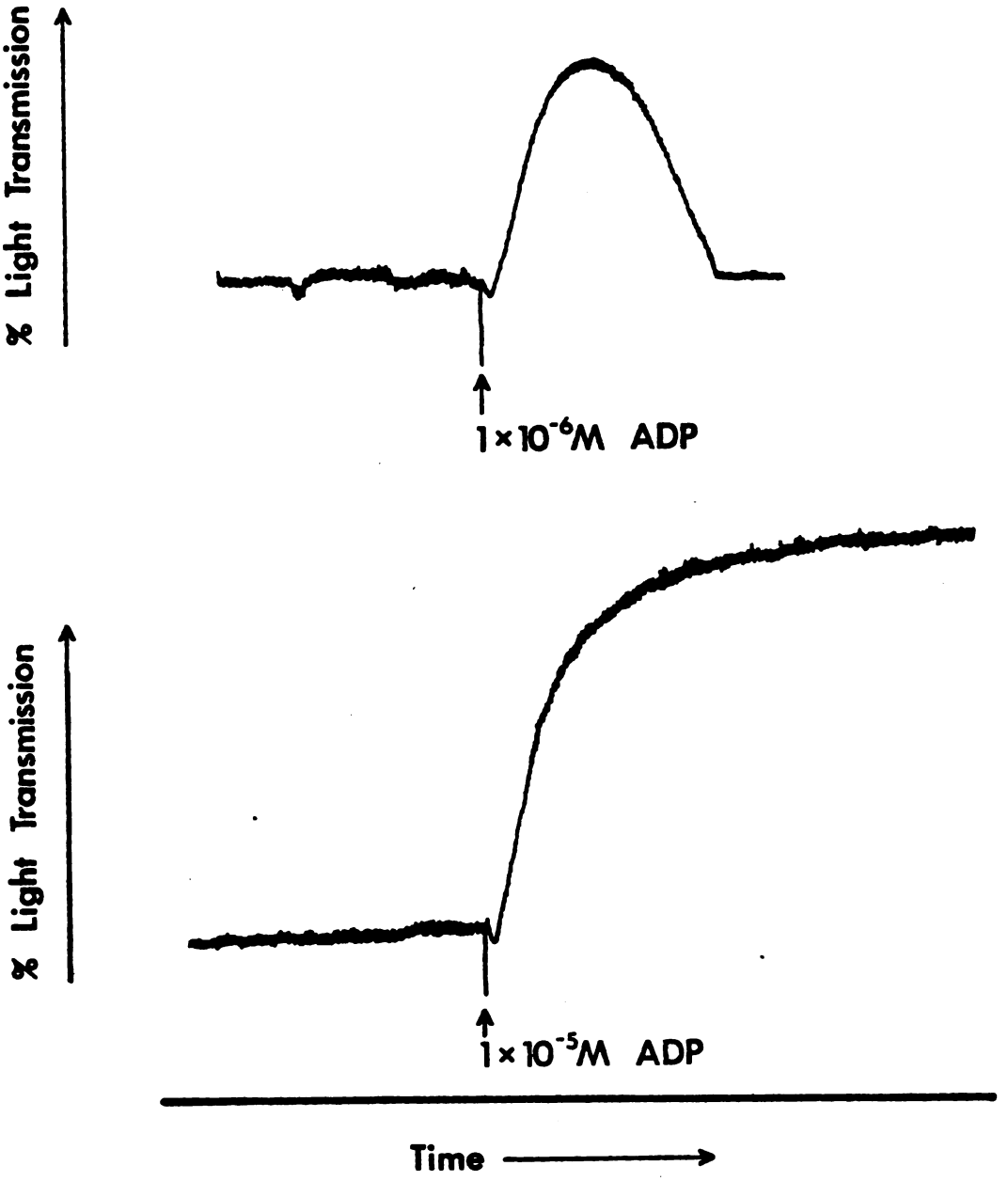


Figure 26. Typical aggregation responses of PRP to ADP. Citrated PRP was prepared from a rat treated with DMF 7 days earlier and aggregations were performed as described in "Materials and Methods."

prostaglandins (notably thromboxane A_2) which cause platelet aggregation to ensue (Smith and Silver, 1976). Typical collagen and arachidonic acid responses in a DMF-treated rat are shown in Figure 27.

Twenty-four hours after MCTP administration, the response of the platelets to three concentrations of ADP, to dog collagen, and to arachidonic acid (AA) were no different from control (Table 20). Four days after MCTP administration, there were also no effects on platelet aggregation due to MCTP treatment (Table 21). Seven days after MCTP, collagen and AA responses were the same as controls but the rate of response and maximum aggregations to ADP were decreased (Table 22). Fourteen days after MCTP, the responses to all of the aggregating stimuli were depressed (Table 23). The delay between injection of aggregating agents and the beginning of aggregation was not altered by MCTP treatment at any of the times examined.

I. Effects of MCTP Treatment on Fawn-hooded Rats

The fawn-hooded (FH) rat strain has a characteristic increase in bleeding time due to a platelet defect (Tschopp and Zucker, 1972). The platelets from FH rats have a decreased content of 5HT and adenine nucleotides in the dense granules and also release less of these compounds when stimulated (Raymond and Dodds, 1975). The platelets have a normal aggregation response *in vitro* to ADP (Raymond and Dodds, 1975) but greatly reduced responses to collagen and arachidonic acid (Wey *et al.*, 1982). Since the thrombocytopenia studies suggested that platelets were involved in MCTP-induced PH, it was of interest to

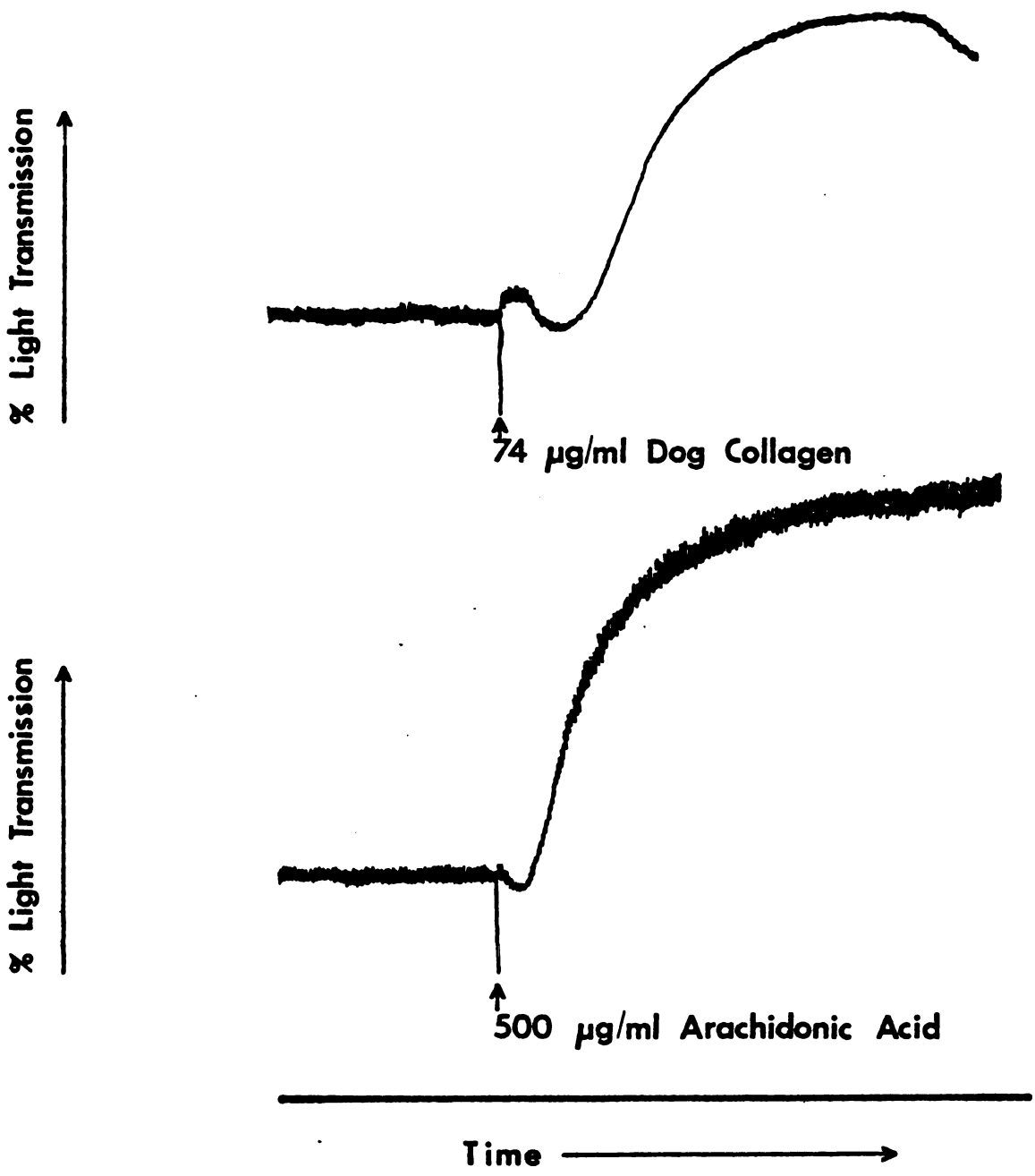


Figure 27. Typical aggregation responses of PRP to dog collagen and arachidonic acid. Citrated PRP was prepared from a rat treated with DMF 7 days earlier and aggregations were performed as described in "Materials and Methods".

TABLE 20

Aggregation Responses of PRP from DMF- or MCTP-treated Rats 24 Hours After Treatment[a]

Aggregating Agent		DMF	MCTP
ADP, $1x10^{-5}M$	Slope	112±4 (11)	121±4 (11)
	Max	62±2 (11)	63±1 (11)
ADP, $2x10^{-6}M$	Slope	101±4 (11)	109±6 (11)
	Max	42±2 (11)	44±2 (11)
ADP, $1x10^{-6}M$	Slope	78±5 (11)	82±7 (9)
	Max	26±3 (11)	23±3 (7)
Dog Collagen, 74 µg/ml	Slope	50, 26-62 (10)	55, 43-71 (10)
	Max	49, 32-62 (10)	55, 49-69 (10)
Arachidonic Acid 500 µg/ml	Slope	82±5 (7)	79±11 (10)
	Max	69±2 (7)	52± 8 (10)
PRP Platelet Count ($x10^{-6}$, mm^{-3})		0.869±0.062 (10)	0.934±0.082 (9)

[a]Rats were given 0.5 ml/kg DMF or 5 mg/kg MCTP in DMF via the tail vein. 24 hours later, rats were bled under ether anesthesia and citrated PRP was prepared. Aggregations were performed by standard methods. "Slope" is the rate of aggregation (%) per minute, "Max" is the maximum % aggregation achieved. All values reported as mean ± S.E.M. except collagen responses which are median, range. Number in parentheses is number in group.

TABLE 21

Aggregation Responses of PRP from DMF- or MCTP-treated Rats 4 Days After Treatment[a]

Aggregating Agent		DMF	MCTP
ADP, $1x10^{-5}M$	Slope	125±1 (6)	125±5 (7)
	Max	66±1 (6)	64±2 (7)
ADP, $2x10^{-6}M$	Slope	112±5 (6)	117±6 (7)
	Max	46±2 (6)	48±2 (7)
ADP, $1x10^{-6}M$	Slope	87±8 (6)	89±7 (7)
	Max	26±4 (6)	26±3 (7)
Dog Collagen, 74 μg/ml	Slope	54, 33-70 (8)	62, 25-83 (10)
	Max	53, 35-59 (8)	59, 18-70 (10)
Arachidonic Acid 500 μg/ml	Slope	75±6 (6)	74±3 (6)
	Max	65±5 (6)	66±4 (6)
PRP Platelet Count ($x10^{-6}$, mm^{-3})		0.927±0.046 (9)	0.934±0.044 (10)

[a]Rats were given 0.5 ml/kg DMF or 5 mg/kg MCTP in DMF via the tail vein. Four days later, rats were bled under ether anesthesia and citrated PRP was prepared. Aggregations were performed by standard methods. "Slope" is the % aggregation per minute, "Max" is the maximum % aggregation achieved. All values reported as mean ± S.E.M. except collagen responses which are median, range. Number in parentheses is number in group.

TABLE 22

Aggregation Responses of PRP from DMF- or MCTP-treated Rats 7 Days After Treatment[a]

Aggregating Agent		DMF	MCTP
ADP, $1x10^{-5}M$	Slope	117±5 (8)	99±5 (10)[b]
	Max	63±2 (8)	53±3 (10)[b]
ADP, $2x10^{-6}M$	Slope	105±6 (8)	82±6 (11)[b]
	Max	44±3 (8)	31±3 (11)[b]
ADP, $1x10^{-6}M$	Slope	73±7 (8)	56±5 (11)
	Max	21±3 (8)	13±2 (11)
Dog Collagen, 74 µg/ml	Slope	36, 27-68 (8)	37, 2-87 (11)
	Max	37, 26-64 (8)	38, 4-65 (11)
Arachidonic Acid 500 µg/ml	Slope	81±5 (7)	75±5 (10)
	Max	68±3 (7)	53±6 (10)
PRP Platelet Count ($x10^{-6}$, mm^{-3})		1.110±0.080 (8)	1.166±0.072 (11)

[a]Rats were given 0.5 ml DMF/kg or 5 mg MCTP/kg in DMF via the tail vein. Four days later, rats were bled under ether anesthesia and citrated PRP was prepared. Aggregations were performed by standard methods. "Slope" is the % aggregation per minute, "Max" is the maximum % aggregation achieved. All values reported as mean ± S.E.M. except collagen responses which are median, range. Number in parentheses is number in group.

[b]Statistically significant difference from DMF group (Student's t-test, $p<0.05$).

TABLE 23

Aggregation Responses of PRP from DMF- or MCTP-treated Rats 14 Days After Treatment[a]

Aggregating Agent		DMF	MCTP
ADP, $1x10^{-5}M$	Slope	119±5 (11)	82±6 (9)[b]
	Max	65±2 (11)	53±3 (9)[b]
ADP, $2x10^{-6}M$	Slope	109±6 (11)	65±7 (9)[b]
	Max	51±3 (11)	28±5 (9)[b]
ADP, $1x10^{-6}M$	Slope	97±7 (10)	36±6 (6)[b]
	Max	35±3 (10)	10±2 (6)[b]
Dog Collagen, 74 µg/ml	Slope	63, 25-93 (9)	36, 3-54 (7)[c]
	Max	64, 29-76 (9)	45, 3-60 (7)[c]
Arachidonic Acid 500 µg/ml	Slope	101±4 (11)	60±7 (9)[b]
	Max	74±1 (11)	47±7 (9)[b]
PRP Platelet Count ($x10^{-6}$, mm^{-3})		1.039±0.039 (11)	1.169±0.080 (9)

[a]Rats were given 0.5 ml DMF/kg or 5 mg MCTP/kg in DMF via the tail vein. Fourteen days later, rats were bled under ether anesthesia and citrated PRP was prepared. Aggregations were performed by standard methods. All values reported as mean ± S.E.M. except collagen responses which are median, range. Number in parentheses is number in group.

[b]Statistically significant difference between DMF and MCTP groups (Student's t-test, $p<0.05$).

[c]Statistically significant difference between DMF and MCTP groups (Rank sum test, $p<0.05$).

compare the effects of MCTP on FH rats with those seen on Sprague-Dawley (SD) rats. MCTP was used rather than MCT because the capacity of FH rats to metabolize MCT was unknown.

The SD rats used as controls were age-matched to the FH rats but the animals grew at different rates, causing the SD rats to be heavier than the FH rats at the time of MCTP dosing (SD average, 288±5 g; FH average, 249±7 g). MCTP treatment caused a decrease in body weight gain in both rat strains (Table 24). Wet lung weight and wet/dry lung weight were increased by MCTP treatment in both strains (Table 24). Dry lung weight was also elevated (data not shown). There were no differences between SD and FH rats in the magnitude of these changes induced by MCTP.

Lung injury was assessed by determining 5HT uptake by the IPL. The ability of lungs of FH rats to remove 5HT from the perfusate was greater than that of lungs from SD rats. The percentage of 5HT in the effluent as metabolite was also higher in FH rats. MCTP decreased removal and metabolism in both strains (Figure 28). The strong influence of strain on 5HT removal precluded conclusions about whether the FH rats were protected from the effects of MCTP on amine removal. The interaction term of the ANOVA comparing rat strains and treatment was not significant, however, suggesting that the effect of MCTP treatment on 5HT removal and metabolism was similar in both strains. The initial perfusion pressures were not different in the DMF-treated animals of either strain. The pressure was elevated in the FH rats treated with MCTP but not in the SD rats similarly treated (data not shown).

TABLE 24

Effects of MCTP on Fawn-Hooded Rats

	Treatment[a]			
	SD/DMF	FH/DMF	SD/MCTP	FH/MCTP
% BW Change	27±1 (8)	23±1 (9)	-2±5[b] (11)	3±5[c] (7)
Wet Lung Wt (g)	1.285±0.054 (8)	1.216±0.040 (9)	3.680±0.504[b] (10)	3.205±0.331[c] (7)
Wet/Dry Lung	5.4±0.1 (8)	5.3±0.1 (9)	8.2±0.6[b] (10)	7.2±0.4[c] (7)
Mortality	0/8	0/9	1/12	4/11

[a]Sprague-Dawley (SD) and fawn-hooded (FH) rats were treated with 0.5 ml DMF/kg or 4 mg MCTP/kg in DMF via the tail vein, 14 days before sacrifice. "% BW Change" is the difference between final and initial body weights expressed as a percentage of the initial body weight. Wet lung weights were obtained after perfusion to determine 5HT removal and metabolism. Mortality is expressed as the number of rats dying prior to 14 days over the number treated. Other values reported as mean ± S.E.M. with the number per group in parentheses.

[b]Significantly different from SD/DMF group (Rank sum test, $p<0.05$).

[c]Significantly different from FH/DMF group (Rank sum test, $p<0.05$).

Figure 28. 5HT removal and metabolism by isolated perfused lungs of fawn-hooded rats after MCTP treatment. Fawn-hooded (FH) or Sprague-Dawley (SD) rats were treated with 5 mg MCTP/kg or 0.5 ml DMF/kg, i.v., 14 days before sacrifice. 5HT removal and metabolism in the IPL were determined as described in "Materials and Methods." Bars are mean ± SEM of groups of 7-11 rats. a = significantly different from SD/DMF group; b = significantly different from FH/MCTP group (2-way factorial ANOVA, lsd test, $p<0.05$).

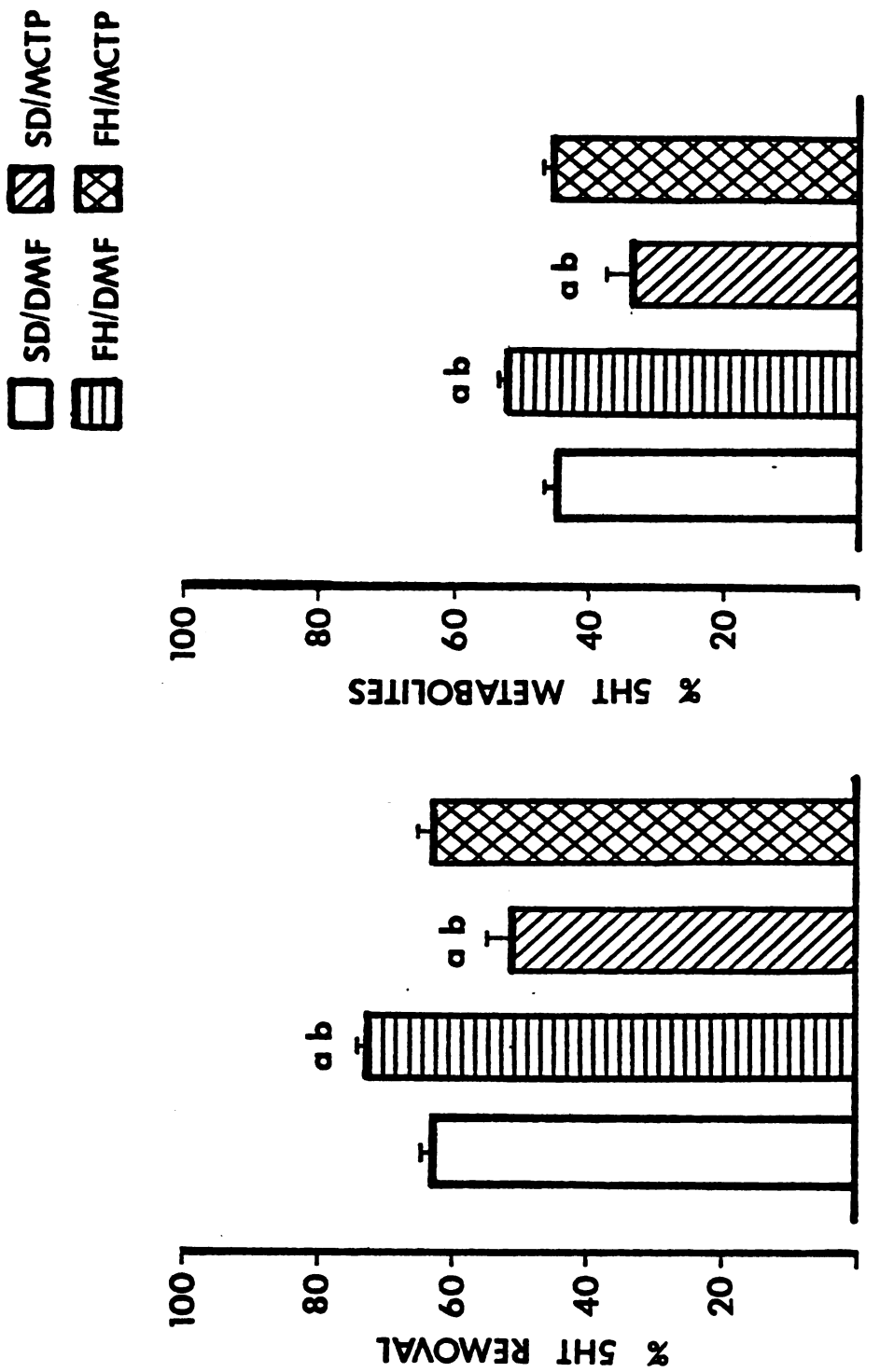

SD/DMF
SD/MCTP
FH/DMF
FH/MCTP
% 5HT REMOVAL
% 5HT METABOLITES
100
80
60
40
20
a b
a b

Figure 28.

Table 25 summarizes the results of heart weight changes caused by MCTP in SD and FH rats. RV/(LV+S) was increased by MCTP treatment in both strains but there was no difference between the SD and FH strains. The ratio of (LV+S) to body weight was increased in the FH rats treated with MCTP. This could reflect left ventricular hypertrophy or the decrease in body weight of this group. To confirm the right ventricular hypertrophy, the ratio of RV to body weight was also compared. Again, right ventricular hypertrophy was observed in the MCTP-treated group of both strains but there was no difference in magnitude between the strains.

Another difference between the SD and FH rats occurred in the blood cell counts (Table 26). The FH rats had a significantly lower platelet count than the SD rats but it was still within the normal range for SD rats of this size (Schalm, 1975). The platelet count was not affected by MCTP treatment. White blood cell count was significantly lower in the FH rats. Differential counts were not done. MCTP treatment increased the circulating white blood cell count in FH rats but not in SD rats. Because FH rats are prone to develop focal glomerular sclerosis from about 4 months of age (Kreisberg and Karnovsky, 1978), BUN and creatinine concentrations were measured to rule out kidney damage as a confounding influence in this study. Neither BUN nor creatinine in the plasma were altered by MCTP treatment (data not shown).

J. Vascular Reactivity in Isolated, Perfused Lungs After MCTP Treatment

Pulmonary hypertension caused by chronic alveolar hypoxia in rats is associated with an increased pulmonary pressor response to

TABLE 25

Effect of MCTP on Heart Weights of Fawn-hooded Rats

	Treatment[a]			
	SD/DMF	FH/DMF	SD/MCTP	FH/MCTP
RV/(LV+S)	0.292±0.009	0.315±0.021	0.417±0.025[b]	0.432±0.036[c]
RV/BW ($x10^3$)	0.61±0.01	0.72±0.06	0.89±0.06[d]	1.23±0.22[e]
(LV+S)/BW ($x10^3$)	2.11±0.05	2.28±0.05	2.13±0.04	2.72±0.28[c]
N	8	9	11	7

[a]Sprague-Dawley (SD) and fawn-hooded (FH) rats were treated with 0.5 ml DMF/kg or 4 mg MCTP/kg in DMF via the tail vein, 14 days before sacrifice. RV is right ventricle weight, (LV+S) is the weight of the left ventricle plus the septum and BW is the final body weight. Values are reported as mean ± S.E.M.

[b]Significantly different from SD/DMF group (2-way factorial ANOVA, lsd test, $p<0.05$).

[c]Significantly different from FH/DMF group (2-way factorial ANOVA, lsd test, $p<0.05$).

[d]Significantly different from SD/DMF group (Rank sum test, $p<0.05$).

[e]Significantly different from FH/DMF group (Rank sum test, $p<0.05$).

TABLE 26

Effect of MCTP on Platelet and White Blood Cell Counts in Fawn-hooded Rats

	Treatment[a]			
	SD/DMF	FH/DMF	SD/MCTP	FH/MCTP
Platelets ($x10^{-6}$, mm^{-3})	1.261±0.550	0.891±0.028[b]	1.296±0.077	0.928±0.074[b]
White Blood Cells (mm^{-3})	16300±2700	3800±300[c]	14200±1700	7500±1300[c,d]
N	7	7	8	3

[a]Sprague-Dawley (SD) and fawn-hooded (FH) rats were treated with 0.5 ml DMF/kg or 4 mg MCTP/kg via the tail vein, 14 days before sacrifice. Values are mean ± S.E.M.

[b]Significantly different from corresponding SD group (2-way factorial ANOVA, lsd test, $p<0.05$).

[c]Significantly different from corresponding SD group (Rank sum test, $p<0.05$).

[d]Significantly different from FH/DMF group (Rank sum test, $p<0.05$).

angiotensin II (AII) (McMurtry et al., 1979; Greenlees et al., 1982). Altered vascular responsiveness to this and other vasoconstrictors could play a role in the development and/or maintenance of increased pulmonary arterial pressure. To explore this possibility in the MCT model of PH, responses to the vasoconstrictors AII and 5HT were examined in blood perfused, isolated lungs from rats with MCTP-induced PH.

Fourteen days after MCTP treatment, AII increased the perfusion pressure of isolated lungs in a dose-dependent manner (Figure 29). At doses of 0.25 or 0.50 μg AII, the responses of lungs from MCTP-treated rats were greater than those of the controls. Figure 30 illustrates a similar pattern for the pulmonary vascular responses to 5HT. At all three doses (12.5, 25, and 50 μg 5HT), lungs from MCTP-treated rats had a greater pressure response than did the controls.

Table 27 contains data comparing the responses of lungs from rats 7 and 14 days after treatment. In contrast to the rats treated 14 days previously, the vasoconstrictor response to AII was not elevated 7 days post-treatment. Response to 5HT was increased at both times, but the effect was greater in the lungs of rats treated 14 days previously.

All of the pulmonary vascular responses were in lungs challenged sequentially with saline, then AII, and finally 5HT. To determine whether the enhanced 5HT response was dependent upon previous challenge with AII, a series of perfused lungs was subjected only to saline and 5HT challenge. These results are summarized in Table 28. Without a previous AII exposure, lungs from rats treated 7 days

Figure 29. Pulmonary pressor responses to angiotensin II (AII) after MCTP treatment. Rats were treated with 5 mg MCTP/kg or 0.5 ml DMF/kg, i.v., 14 days before sacrifice. Pressor responses were determined as described in "Materials and Methods." Points are mean ± SEM of groups of 4-6 rats. *Significantly different than DMF group at same dose (Rank sum test, $p<0.05$).

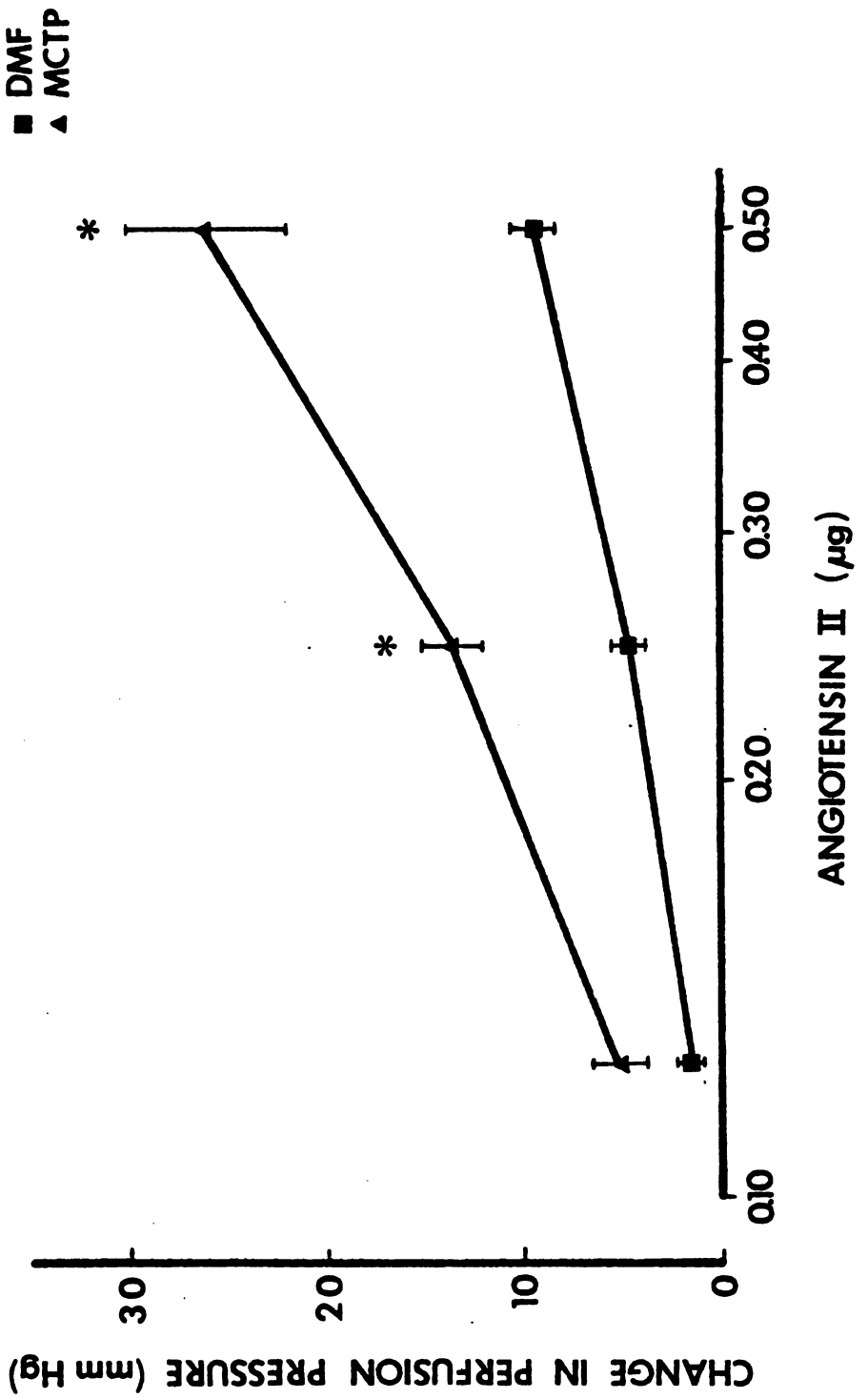

■ DMF
▲ MCTP
CHANGE IN PERFUSION PRESSURE (mm Hg)
0
10
20
30
0.10
0.20
0.30
0.40
0.50
ANGIOTENSIN II (μg)
*
*

Figure 29.

Figure 30. Pulmonary pressor responses to 5HT after MCTP treatment. Rats were treated with 5 mg MCTP/kg or 0.5 ml DMF/kg, i.v., 14 days before sacrifice. Pressor responses were determined as described in "Materials and Methods." Points are mean ± SEM of groups of 4-6 rats. *Significantly different than DMF group at same dose (Rank sum test, $p<0.05$).

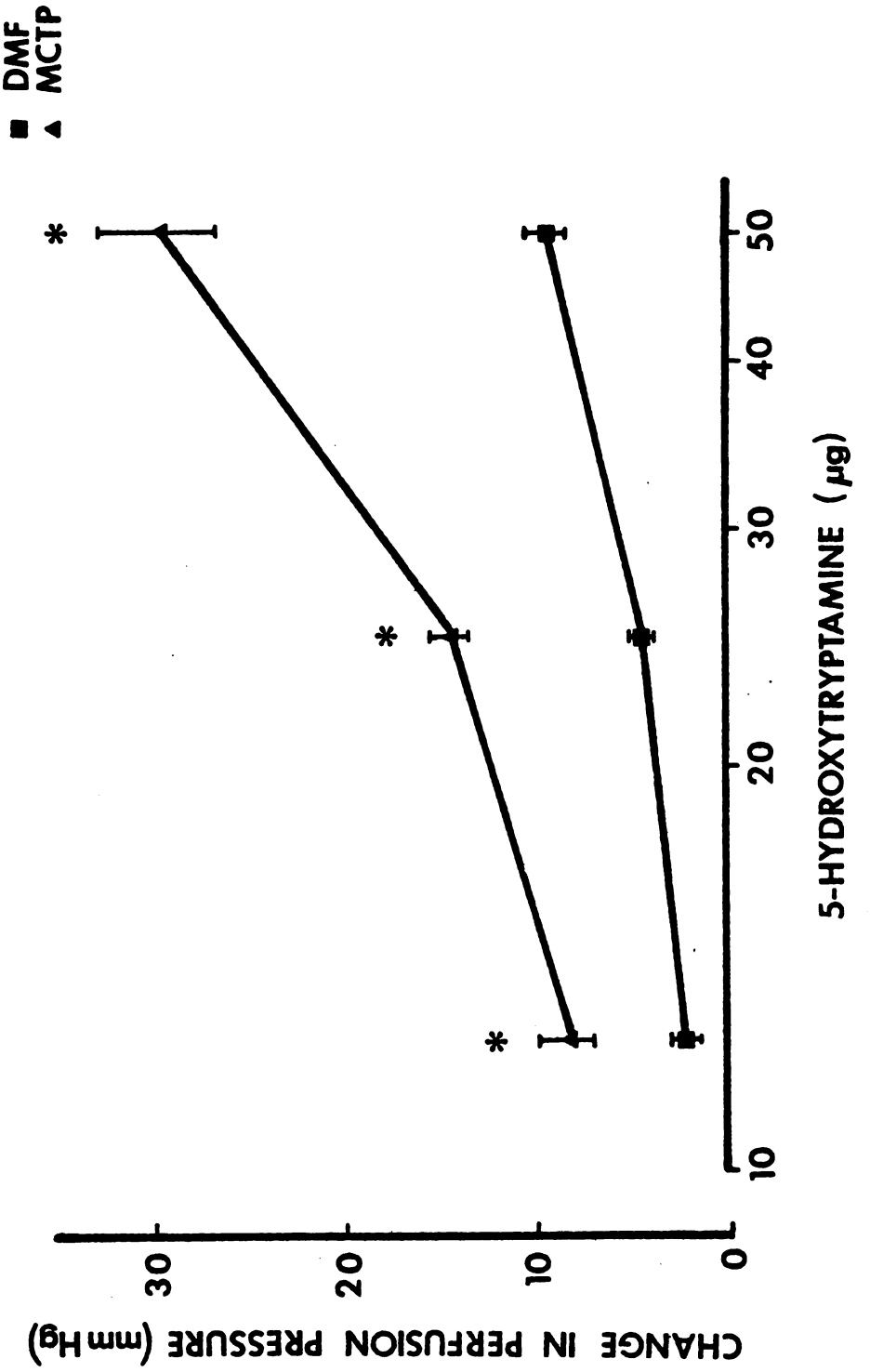

DMF
MCTP
CHANGE IN PERFUSION PRESSURE (mm Hg)
0
10
20
30
5-HYDROXYTRYPTAMINE (μg)
10
20
30
40
50
*
*
*

Figure 30.

TABLE 27

Comparison of Pulmonary Responses 7 and 14 Days After MCTP Administration[a]

	Baseline Perfusion Pressure (mmHg)	ΔPressure, 0.25 μg AII	Paired Differences[b]	ΔPressure, 25 μg 5HT	Paired Differences[b]	Wet Lung Weight (g)
7 Day						
DMF	12(11-13)	7(3-10)	2(-2-6)	8(5-10)	5(2-8)	1.560±0.032
MCTP	13(11-14)	9(4-12)		13(10-16)[c]		2.474±0.283[e]
14 Day						
DMF	12(11-13)	5(3-8)	9(3-15)[d]	4(2-5)	10(8-13)[d]	1.496±0.058
MCTP	16(13-18)[c]	13(7-20)[c]		14(10-18)[c]		2.645±0.172[e]

[a]Rats were treated with 0.5 ml DMF/kg or 5 mg MCTP/kg and killed 7 days later as described in "Materials and Methods". Wet lung weight is expressed as mean ± S.E.M. All other data are presented as mean and range. N = 6-7.

[b]Mean and range of the differences in response between paired DMF and MCTP lungs perfused on the same day.

[c]Statistically significant difference from DMF group (Rank sum test, $p<0.05$).

[d]Statistically significant difference between 7 day and 14 day groups (Signed rank test, $p<0.05$).

[e]Statistically significant difference from DMF group (Student's t-test, $p<0.05$).

TABLE 28

Pulmonary Vascular Responses to 5HT Challenge Only, 7 and 14 Days After MCTP

	Baseline Perfusion Pressure (mmHg)	ΔPressure After 25 μg 5HT (mmHg)	Wet Lung Weight (g)
7 Day			
DMF	12(10-14)	6(5-7)	1.497±0.056
MCTP	13(11-16)	9(5-17)	2.659±0.048[c]
14 Day			
DMF	12(12-12)	3(2-4)	1.329±0.031
MCTP	15(14-17)[b]	6(4-9)[b]	2.728±0.309[c]

[a]Rats were treated with 0.5 ml DMF/kg or 5 mg MCTP/kg and killed 7 or 14 days later as described in Materials and Methods. Data for baseline perfusion pressure and pressure after 5HT are expressed as mean and range. Wet lung weight is presented as mean ± S.E.M. N = 4-5.

[b]Statistically significant difference from DMF group (Rank sum test, $p<0.05$).

[c]Statistically significant difference from DMF group (Student's t-test, $p<0.05$).

earlier with MCTP did not have an increased pressure response to 25 μg 5HT. Lungs from rats treated with MCTP 14 days earlier did have an enhanced response to 25 μg 5HT but the pressure elevations were smaller than those elicited by 5HT after the lungs had been exposed to AII.

To examine the possibility that the increased response to 5HT 14 days after MCTP was related to decreased removal of 5HT from the pulmonary vasculature, a set of lung perfusions was done in which the perfusion medium contained 10^{-5}M imipramine. Imipramine at this concentration reduces removal of perfused 5HT by the isolated rat lung about 95% (Hilliker and Roth, unpublished observations; Junod, 1972). The pressure responses to 0.25 μg AII and 25 μg 5HT in lungs of 14 day vehicle- or MCTP-treated rats were not increased by the inclusion of imipramine in the perfusion medium (Table 29).

K. Effects of Drug Treatments on MCTP Toxicity

As another approach to identifying the mechanisms involved in MCT induction of PH, a series of exploratory experiments were done in which the effect of various drugs on MCTP toxicity were examined. MCTP rather than MCT was used to induce PH to avoid possible confounding of the results by drug effects on metabolic activation of MCT.

Hydralazine hydrochloride (HD) is a vasodilator which has been used with some success in cases of human primary pulmonary hypertension (Fein and Frishman, 1980). Besides acting as a vasodilator, HD also inhibits platelet aggregation and thromboxane synthesis (Greenwald et al., 1978). The dose used in this experiment was based on

TABLE 29

Effect of Imipramine on Pulmonary Vascular Responses to AII and 5HT

Treatment[a]	N	Baseline Perfusion Pressure (mmHg)	ΔP, 0.25 μg AII (mmHg)	ΔP, 25 μg 5HT (mmHg)
DMF	6	12(11-13)	5(3-8)	4(2-5)
DMF/IMIP	5	13(11-14)	4(3-7)	4(2-6)
MCTP	6	16(13-18)[b]	13(7-20)[b]	14(10-18)[b]
MCTP/IMIP	5	16(14-18)[c]	9(5-16)[c]	8(4-16)[c,d]

[a]Rats were treated with either 0.5 ml DMF/kg or 5 mg MCTP/kg 14 days before sacrifice. Imipramine (IMIP, 10^{-5}M) was added to the perfusion medium for the indicated groups. Values reported as mean and range.

[b]Statistically significant difference from DMF group (2-way factorial ANOVA, lsd test, $p<0.05$).

[c]Statistically significant difference from DMF/IMIP group (2-way factorial ANOVA, lsd test, $p<0.05$).

[d]Statistically significant difference from MCTP group (2-way factorial ANOVA, lsd test, $p<0.05$, significant ANOVA interaction term).

studies in retired breeder rats in which a dose of 3 mg HD/kg normalized their hyperreactive platelets (Burns and Saunders, 1979). This dose of HD, administered daily, reduced the right ventricular hypertrophy and the increase in lavage fluid protein caused by MCTP (Table 30). Body weight and lung weight changes were not affected by HD.

Glucocorticoids have a wide spectrum of pharmacologic actions (Haynes and Larner, 1975). They are frequently used for their anti-inflammatory and immune suppressant effects. Since inflammatory changes are a prominent characteristic of the response to MCT, it was of interest to test the effects of dexamethasone (DX) on MCTP toxicity. DX also inhibits release of arachidonic acid from phospholipids by inhibiting phospholipase A_2 (Flower, 1978). The dose used was 0.027 mg DX/kg, daily. This dose was anti-inflammatory in rats and had minimal effects on adrenal, thymus and body weight in rats treated for 4 days (Dr. Elliott Collins, Schering Corp., personal communication). When administered for 14 days, however, this dose of DX caused a decrease in the body weight gain compared to saline-treated controls (Table 31). There was no further decrease in weight gain in the DX/MCTP-treated animals. Dexamethasone prevented the right ventricular hypertrophy usually seen after MCTP treatment. The drug also reduced the increase in wet lung to body weight ratio and in lavage fluid protein concentration. Treatment with DX did not alter lavage fluid LDH activity.

Sulfinpyrazone (SP) is known mainly as a uricosuric agent useful in the treatment of gout but it is also a reversible inhibitor of the cyclooxygenase enzyme in the prostaglandin synthesis pathway (Fuster

TABLE 30

Effects of Hydralazine on MCTP Toxicity

	Treatment[a]			
	Sal/DMF	HD/DMF	Sal/MCTP	HD/MCTP
ΔBW (g)	103±8	94±6	73±16	84±4
Wet Lung Wt (g)	1.176±0.058	1.195±0.019	1.599±0.121[b]	1.507±0.133
Lavage Fluid LDH (U/100 ml)	1.6(1.5-1.9)	1.5(0.1-1.9)	5.6(2.3-47.1)[b]	4.6(2.2-8.4)[c]
Lavage Fluid Protein (mg/ml)	0.09±0.01	0.07±0.00	0.70±0.10[b]	0.34±0.10[c,d]
RV/(LV+S)	0.320±0.012	0.320±0.007	0.419±0.025[b]	0.350±0.003[c,d]

[a]Rats were treated daily with 3 mg hydralazine HCl (HD)/kg or an equivalent volume of saline (Sal). After 3 days of treatment, rats received 0.5 ml DMF/kg or 5 mg MCTP/kg in DMF via the tail vein. The rats were sacrificed 14 days after MCTP treatment. "ΔBW" is the difference in body weight between the day of sacrifice and the day of MCTP treatment. Lavage fluid LDH activity and protein concentration were determined as described in "Methods". Values reported as mean ± S.E.M. except for lavage LDH which is median (range). N = 6-8.

[b]Significantly different from Sal/DMF group (Rank sum test, $p<0.05$).

[c]Significantly different from HD/DMF group (Rank sum test, $p<0.05$).

[d]Significantly different from Sal/MCTP group (Rank sum test, $p<0.05$).

TABLE 31

Effects of Dexamethasone on MCTP Toxicity

	Treatment[a]			
	Sal/DMF	DX/DMF	Sal/MCTP	DX/MCTP
ΔBW (g)	115±14	61±3[b]	41±22[b]	53±6
Wet Lung/BW (x1000)	3.7±0.1	3.6±0.1	9.4±1.7[b]	4.7±0.4[c,d]
Lavage Fluid LDH (U/100 ml)	1.7±0.1	1.9±0.1	14.4±2.9[b]	14.9±2.4[c]
Lavage Fluid Protein (mg/ml)	0.04±0.01	0.06±0.01	2.19±0.87[e]	0.29±0.05[f,g]
RV/(LV+S)	0.326±0.007	0.284±0.010[b]	0.385±0.022[b]	0.310±0.007[d]

[a]Rats were treated daily with 0.027 mg dexamethasone (DX)/kg or 0.5 ml saline (Sal)/kg, i.p. After 3 days of treatment, rats received 0.5 ml DMF/kg or 5 mg MCTP/kg in DMF via the tail vein. The rats were sacrificed 14 days after MCTP treatment. "ΔBW" is the difference in body weight between the day of sacrifice and the day of MCTP treatment. Wet lung/body weight ratios were calculated using the body weight at sacrifice. Lavage fluid LDH activity and protein concentration were determined as described in "Materials and Methods". Values reported as mean ± S.E.M. N = 7-8.

[b]Significantly different from Sal/DMF group (Rank sum test, $p<0.05$).

[c]Significantly different from DX/DMF group (Rank sum test, $p<0.05$).

[d]Significantly different from Sal/MCTP group (Rank sum test, $p<0.05$).

[e]Significantly different from Sal/DMF group (2-way factorial ANOVA, lsd test, $p<0.05$).

[f]Significantly different from DX/DMF group (2-way factorial ANOVA, lsd test, $p<0.05$).

[g]Significantly different from Sal/MCTP group (2-factorial ANOVA, lsd test, $p<0.05$).

and Chesebro, 1981). Platelet prostaglandin synthesis was decreased without a concomitant decrease in vascular prostacyclin synthesis in rats by SP (Livio et al., 1980). Treatment with SP also increased platelet survival time in rats with surgically damaged aortas (Wilkinson et al., 1979). In this study 2 doses of 60 mg SP/kg per day were effective in increasing platelet survival time. Such a treatment regimen did not alter platelet aggregation responses to ADP, collagen or arachidonic acid in our hands, however. Increasing the dose to 100 mg SP/kg twice a day reduced the responses to arachidonic acid by approximately 50%. Higher doses were lethal. The 100 mg/kg dose prevented right ventricular hypertrophy in MCTP-treated rats but did not alter any of the other indexes of lung injury measured (Table 32).

Aspirin (acetylsalicylic acid; ASA) is another drug which affects platelets via effects on prostaglandin synthesis. In contrast to SP, aspirin irreversibly inhibits prostaglandin cyclooxygenase by acetyl--ating the enzyme (Roth and Majerus, 1975). Both platelet and vascular wall prostaglandin synthesis are affected. These effects can be separated by judicious administration of the drug. Endothelial cells can recover the ability to synthesize PGI_2 relatively rapidly, within 18-24 hours (Jaffe and Weksler, 1979). Platelets have no protein synthetic capability and must thus be replaced before TXA_2 synthesis commences at a detectable level (Roth and Majerus, 1977). This difference was exploited by treating rats at 5 day intervals with 200 mg ASA/kg. A 200 mg/kg dose has been reported to inhibit 5HT release by platelets in response to collagen for 2-3 days and to inhibit malondialdehyde production (an index of prostaglandin synthesis) for up to

TABLE 32

Effects of Sulfinpyrazone on MCTP Toxicity

	Treatment[a]			
	Veh/DMF	SP/DMF	Veh/MCTP	SP/MCTP
ΔBW (g)	83±8	23±12[b]	57±20	46±6
Wet Lung Wt (g)	1.090±0.056	0.991±0.063	1.740±0.257[c]	1.479±0.163[d]
Lavage Fluid LDH (U/100 ml)	1.7(1.4-2.1)	1.5(1.1-2.2)	7.1(2.2-20.9)[b]	14.0(1.5-31.2)
Lavage Fluid Protein (mg/ml)	0.08±0.01	0.06±0.01	1.16±0.56[c]	0.26±0.08[d]
RV/(LV+S)	0.302±0.011	0.308±0.008	0.367±0.011[c]	0.323±0.014[e]

[a]Rats were treated two times daily with 100 mg sulfinpyrazone (SP)/kg or 1 ml propylene glycol (Veh)/kg, i.p. After 3 days of treatment, rats received 0.5 ml DMF/kg or 5 mg MCTP/kg in DMF via the tail vein. The rats were sacrificed 14 days after MCTP treatment. "ΔBW" is the difference in body weight between the day of sacrifice and the day of MCTP treatment. Lavage fluid LDH activity and protein concentration were determined as described in "Methods". Values reported as mean ± S.E.M. N = 5-6. Lavage LDH reported as mean (range).

[b]Significantly different from Veh/DMF group (Rank sum test, $p<0.05$).

[c]Significantly different from Veh/DMF group (2-way factorial ANOVA, lsd test, $p<0.05$).

[d]Significantly different from SP/DMF group (2-way factorial ANOVA, lsd test, $p<0.05$).

[e]Significantly different from Veh/MCTP group (2-way factorial ANOVA, lsd test, $p<0.05$).

4 days (Gordon et al., 1976). However, this dose of ASA administered at 5 day intervals in conjunction with MCTP did not alter right ventricular hypertrophy or any of the indexes of lung injury examined (Table 33).

Metergoline is a 5HT antagonist in both central and peripheral tissues (Sastry and Phillis, 1977). It inhibits both platelet aggregation in response to ADP and 5HT potentiation of ADP aggregation in rat platelets in vitro (Doni, 1980). The dose chosen for this study inhibits 5HT neuronal transmission in the brain (Demarest and Moore, 1981). This dose did not affect changes in body weight gain, wet lung weight or right ventricular hypertrophy in MCTP-treated rats, however (Table 34).

TABLE 33

Effects of Aspirin on MCTP Toxicity[a]

	Veh/MCTP	ASA/MCTP
ΔBW (g)	10±15	32±14
Wet Lung Wt (g)	2.583±0.187	2.719±0.218
Lavage Fluid LDH (U/100 ml)	21.2±3.4	15.3±2.3
Lavage Fluid Protein (mg/ml)	3.94±0.87	3.64±0.88
RV/(LV+S)	0.377±0.017	0.369±0.013

[a]Rats were treated every 5 days with 200 mg acetylsalicylic acid (ASA)/kg or 4 ml 0.5% methyl cellulose (Veh)/kg, p.o. 24 hours after the first dose of ASA or Veh, the rats were treated with 5 mg MCTP/kg in DMF via the tail vein. Fourteen days after MCTP treatment the rats were sacrificed. "ΔBW" was the change in body weight between the day of sacrifice and the day of MCTP treatment. Lavage fluid LDH activity and protein concentration were determined as described in Materials and "Methods". Values reported as mean ± S.E.M. There were no significant differences between the two groups (Student's t-test, $p<0.05$). N = 11-14.

TABLE 34

Effects of Metergoline on MCTP Toxicity

	Treatment[a]			
	Veh/DMF	MTG/DMF	Veh/MCTP	MTG/MCTP
ΔBW (g)	63±4	76±2[b]	26±8[b]	52±9[c]
Wet Lung Weight (g)	1.702±0.135	1.416±0.080	2.214±0.123[d]	1.995±0.128[e]
RV/(LV+S)	0.290±0.010	0.284±0.007	0.354±0.017[d]	0.341±0.012[e]

[a]Rats were treated daily with 1 mg metergoline (MTG)/kg or 1 ml 0.5% methyl cellulose (Veh)/kg, i.p. Four hours after the first dose of Veh or MTG, the rats received 0.5 ml DMF/kg or 5 mg MCTP/kg in DMF. N=6.

[b]Significantly different than Veh/DMF group (Rank sum test, $p<0.05$).

[c]Significantly different than MTG/DMF group (Rank sum test, $p<0.05$).

[d]Significantly different than Veh/DMF group (2-way factorial ANOVA, lsd test, $p<0.05$).

[e]Significantly different than MTG/DMF group (2-way factorial ANOVA, lsd test, $p<0.05$).

DISCUSSION

A. Dose Response to a Single Injection of MCT

A single subcutaneous injection of MCT results in the same pulmonary changes as have been observed after feeding. The doses tested reduced the gain in body weight over the two week experimental period and increased the wet lung weight, both characteristics common to MCT intoxication (Lalich and Merkow, 1961; Huxtable et al., 1978; Roth et al., 1981). A single injection of MCT caused a decrease in 5HT removal and metabolism by the isolated, perfused lung as has been described by others (Gillis et al., 1978; Huxtable et al., 1978; Roth et al., 1981). This route of administration resulted in minimal hepatic damage. Even at the highest dose tested there was only a slight elevation in plasma GPT activity. When the liver was seriously compromised by pyrrolizidine intoxication in earlier studies, SGPT activity was much larger, approximately 10 times control values (McLean, 1964) as opposed to double control values as observed in this study. A single injection of MCT can also lead to pulmonary hypertension as evidenced by the right ventricular hypertrophy observed 14 days after 105 mg/kg. A 60 mg/kg injection did not cause RVH in this study but previous studies showed significant increases in pulmonary arterial or right ventricular systolic pressure and right ventricular weight by 14 days after this dose (Ghodsi and Will, 1981; Kay et al.,

1982). Feeding of MCT requires a somewhat longer period for the development of PH. Increased pulmonary arterial pressure has been reported as early as 14 days after beginning treatment but right ventricular hypertrophy was not significant until 21-28 days (Meyrick and Reid, 1979; Meyrick et al., 1980; Roth et al., 1981).

A treatment regimen based on a single injection of MCT has several advantages over feeding the compound. An injection delivers a defined dose to each animal in the study, eliminating variability due to differing food or water intakes of individual rats. Using a defined dose provides an opportunity to examine dose response relationships in a quantitative manner if desired. Treating animals with one injection rather than chronic, repeated exposures also allows construction of temporal relationships between exposure to the toxin, its concentration, tissue injury and the detection of physiological effects. Such temporal relationships may be helpful in the determination of the etiology of pulmonary hypertension in this model.

Based on the dose response studies described, 105 mg MCT/kg was chosen for further study. This dose consistently caused right ventricular hypertrophy by 14 days after injection with minimal mortality. Pulmonary effects were large enough to be detected reliably and hepatic damage was minimal. The next step was to determine how these effects developed in the 14 day period between injection and sacrifice.

B. Development of Responses to a Single Injection of MCT

Responses to MCT injection were examined 2, 5, 10 and 14 days after injection. The earliest changes seen were a decrease in the

circulating platelet count and an increase in plasma GPT activity 2 days after the MCT injection. The decrease in circulating platelets may be due to sequestration by the spleen or liver or to lodging in the capillary bed of the lung. A frequently noted morphological feature of MCT administration has been the occurrence of platelet-containing thrombi in the lungs (Lalich and Ehrhart, 1962; Turner and Lalich, 1965; Merkow and Kleinerman, 1966; Hayashi and Lalich, 1967; Smith and Heath, 1978; Meyrick and Reid, 1982). Such thrombi have been observed as soon as 4 hours after a single injection of MCT (Valdivia et al., 1967). Meyrick and colleagues (1980) observed no effects of MCT on platelet counts after feeding Crotalaria spectabilis seeds. This difference in results could be due to the difference in treatment regimens. Animals receiving a single injection were probably exposed to a higher concentration of MCT and for a shorter time than rats fed low doses chronically, potentially altering the platelet effects. In addition, the seeds contain many compounds besides MCT, some of which might modify the effects on platelets. However, it is not completely clear that feeding the seeds did not cause at least a transient drop in platelet count. In this study the investigators examined animals at weekly intervals, starting at 7 days, and may have missed a transient effect. Also, the platelet counts at the various intervals were not presented. The text stated that "the platelet counts of the Crotalaria-fed animals and controls...were within the normal range (590,000-1,200,000/mm^3)." Our experience and that of other sources is that the normal platelet count in Sprague-Dawley rats of this age ranges from 800,000-1,200,000/mm^3 (Schalm, 1975). If the

rats with the lower platelet counts in this study were the Crotalaria-fed animals, these investigators may have observed the rats developing or recovering from thrombocytopenia without realizing it.

The decrease that we observed in the platelet count persisted at least until 10 days after injection. By 14 days after treatment the platelet count was elevated. This may be an "overshoot" due to the response of normal physiological control mechanisms to the prolonged decrease in circulating platelets. Alternatively, it might reflect a response to an increased demand for platelets, e.g., by progressive endothelial cell damage.

Plasma GPT activity has been used as a general index of acute hepatocellular injury. Increases are presumed to be due to the release of the transaminase into the blood from the cytosol of damaged and/or necrotic cells. A single injection of MCT caused an increase in plasma GPT activity above control values only at early times following the 105 mg/kg dose. The short duration of elevated transaminase activity may be related to the rapid elimination of MCT. Hepatic injury may only occur when MCT or its metabolites are available in the blood. At times beyond which MCT concentrations are low or non-detectable (Hayashi, 1966; Mattocks, 1972; Allen et al., 1972), GPT activity in the blood is normal. It should be noted that GPT elevations were again relatively small, suggesting the presence of only minimal hepatic injury. The absence of severe pathological changes in the livers of treated rats was consistent with the low GPT activity.

The removal and metabolism of 5HT by the isolated, perfused lung was examined as an index of pulmonary damage. Three major pieces of

information are obtained from the IPL preparation. The "% 5HT removal" describes the percentage of 5HT taken up by the lung regardless of its subsequent fate. The "% 5HT metabolites" represents the percentage of the perfused 5HT which is in the form of metabolites in the effluent. The perfusion pressure was monitored throughout each perfusion and provided a quality control monitor for the perfusion as well as information about the lung vasculature. An increase in perfusion pressure over the duration of the perfusion is usually a sign of edema development within the lung. Any preparation which exhibited a change in perfusion pressure during 5HT or NE removal determinations was excluded from the analysis. The baseline perfusion pressure also provided information about the pulmonary vasculature, acting as an index of pulmonary vascular resistance. Typically, rats with established PH (i.e., right ventricular hypertrophy) also exhibited an increased baseline or initial perfusion pressure. This probably reflects the extensive medial thickening in the pulmonary vessels.

The uptake of 5HT from the circulation is a function of pulmonary endothelial cells and may be a sensitive indicator of early lung vascular injury (see Introduction). 5HT removal by the pulmonary circulation was not changed at 2 days after MCT injection but was significantly decreased by 5 days. It continued to decrease with time, suggesting that the damage to endothelium progressively increased. The percentage of 5HT in the effluent as metabolites was also decreased at these times. Since pulmonary monoamine oxidase activity was not altered by MCT treatment (Gillis et al., 1978; Huxtable et al., 1978); the decrease in metabolites probably reflects the decrease in uptake.

The removal of another biogenic amine, norepinephrine, was also decreased by MCT treatment. This experiment was undertaken to clarify a difference between two previous reports and to further compare MCT-induced PH to human PPH. Gillis and coworkers (1978) reported a 60% decrease in NE removal by the IPL after 21 days of feeding MCT in the drinking water (22 μg/ml). Shortly thereafter, a report that 21 days of MCT in the drinking water did not reduce NE removal was published (Huxtable et al., 1978). Aspects of both studies could explain the apparent discrepancy. The initial study (Gillis et al., 1978) drew its conclusion from only 3 MCT-treated rats and, while the results seemed to be consistent and of reasonable magnitude, extrapolation from a sample size of 3 to an entire population is risky. Huxtable and colleagues (1978) based their conclusions on perfusions performed at room temperature. The NE uptake process is temperature-sensitive and this may have confounded the interpretation of the results since the fraction of NE taken up into lung by carrier-mediated transport is diminished at lowered temperatures (Iwasawa et al., 1973). Our studies indicated that NE removal but not metabolism was decreased by MCT injection, even at only 14 days after treatment. This observation further strengthens the validity of MCT-induced PH as a model for human PH since decreased NE removal has also been described in patients with increased pulmonary vascular resistance (Sole et al., 1979; Gewitz et al., 1982).

There was no evidence of pulmonary hypertension in the rats examined 2, 5, or 10 days after a single injection of MCT. By 14 days, however, two indirect measurements suggested that pulmonary

arterial pressure had become elevated. First, the inflow perfusion pressure was increased in the isolated lung preparations from MCT-treated rats suggesting increased pulmonary vascular resistance. Elevation of pulmonary vascular resistance may occur earlier than 14 days in vivo, but in lungs perfused at low flows such increases might not be detectable. Secondly, right ventricular hypertrophy, which develops in response to increased pulmonary arterial pressure (Fulton et al., 1952; Ghodsi and Will, 1981), did not occur until between 10 and 14 days after treatment. The temporal relationship between increased pulmonary arterial pressure and the development of right ventricular hypertrophy is not entirely clear (Meyrick et al., 1980; Ghodsi and Will, 1981; Kay et al., 1982). An interesting aspect of the right ventricular hypertrophy is the speed with which it develops. Regardless of the treatment regimen used for MCT administration, the earliest detection of RVH has been 12 days after injection or initiation of feeding (Huxtable et al., 1977; Kay et al., 1982). Ten days after administration there has been no evidence of an increase in the right ventricle weight (this study, Kay et al., 1982) suggesting that the hypertrophy develops in just 2-4 days.

C. Synthesis of MCTP

The synthesis of MCTP was undertaken to perform experiments for which the metabolite would be more useful than the parent compound. These experiments fall basically into two categories: Studies to determine whether the metabolite can interact in vitro with tissues to cause immediate damage and studies to examine the effects of other

manipulations (e.g., drug treatments, thrombocytopenia) on MCT toxicity. Since these manipulations might affect the conversion of MCT to MCTP we wished to use MCTP directly. Thus, the primary goal was to produce a product which would reproducibly induce PH and lung injury in rats comparable to MCT administration. A full discussion of the biological effects of MCTP is presented in the next section. Ideally, when administering a compound which produces toxic effects, its purity and, if possible, what compounds are present as contaminants should be known. Because of the extremely reactive nature of the MCTP, it has not yet been possible to estimate its purity quantitatively or to identify potential contaminants. At this point, it is reasonable to conclude that the product is largely MCTP based on the mass spectrometry of the product and the largest component after TLC separation. Because of apparent interaction between the TLC solvent systems and the product an exact estimate of purity is not possible, however.

D. Development of Responses to a Single Injection of MCTP

Another study from our laboratory (Bruner et al., 1983) has characterized the development of MCTP-induced PH. In this study, various cardiopulmonary parameters were evaluated at 3, 5, 7, 10 and 14 days after a single injection of MCTP. Control rats gained weight throughout the study, but the MCTP-treated rats had stopped gaining by 7 days after treatment. Lung injury, in the form of elevated lavage fluid LDH activity, was present by 4 days after treatment and remained increased through 14 days after treatment. Protein concentration in the lavage fluid was also elevated at 5, 7, 10, and 14 days.

Pulmonary arterial pressure was measured in closed chest, anesthetized rats. There was no difference between control and treated groups at 3 and 5 days, but pulmonary arterial pressure was significantly increased 7, 10 and 14 days after treatment in the MCTP groups. Right ventricular hypertrophy was not evident at any of the time points examined except 14 days, at which point it was comparable to that observed after a single injection of MCT (105 mg/kg, s.c.).

My experiments were designed to determine when and if MCTP affected 5HT uptake and metabolism by the IPL. The effects of MCTP on body weight gain and lung weight were similar to those seen previously (Bruner et al., 1983). Right ventricular hypertrophy was also evident at 14 days after treatment. The pattern of effects on 5HT removal/metabolism was quite different after MCTP than after a single injection of MCT. Removal of 5HT was decreased by 5 days after MCT treatment but not until between 7 and 14 days after MCTP treatment. The amount of 5HT in the effluent as metabolites was decreased at 5, 7 and 14 days after MCTP administration. The significance of this is unclear. A decrease in the percent metabolites without a change in the percent removal would suggest that MAO activity was decreased by MCTP in contrast to MCT which does not affect MAO activity (Gillis et al., 1978). MAO activity in lung homogenates from MCTP-treated rats should be determined to confirm this hypothesis.

In striking contrast to MCT, MCTP did not alter the circulating platelet count at times ranging from 30 minutes to 14 days after MCTP injection. The reason for this difference between MCT and MCTP is unclear but may lie in the primary difference between the two compounds. MCT requires metabolic activation by the liver to cause PH.

Metabolites other than MCTP are undoubtedly produced from MCT and one of these may be responsible for the platelet effect. Another possibility is that the platelet decrease is the result of a transient depression of the bone marrow. The pyrrole metabolites of the pyrrolizidine alkaloids have been shown to be anti-mitotic (Culvenor et al., 1969; Hsu et al., 1973b) and could thus halt cell division in rapidly dividing cells such as the bone marrow. Unfortunately, only platelet counts were measured in the MCT study, so it is not possible to support or rule out this theory by examining whether general bone marrow suppression had occurred or whether the effect was limited to platelets. The platelets may also be sequestered in the liver in response to MCT-induced hepatic injury. The results of these experiments imply that the decreased platelet count was not necessary for the development of MCT-induced pulmonary hypertension.

E. In Vitro Exposure of Lung Slices to MCT Metabolites

The first approach to determining whether MCTP could interact with lung cells and cause injury directly was to incubate lung slices with MCT metabolites and measure the effects of this incubation on 5HT uptake. The interpretation of uptake in a lung slice system is complicated by the fact that a tissue slice has many surfaces available for uptake that are not exposed in the isolated, perfused lung. Many cell types are exposed directly to the medium and present potential sites of non-specific uptake. However, 5HT uptake by isolated endothelial cells is much greater than by other cell types including fibroblasts and red blood cells (Shepro et al., 1975). In addition,

nonspecific uptake by these other cell types is not inhibited by imipramine. Most of the uptake in both the slice and IPL preparations in our experiments was inhibited by imipramine, suggesting that specific uptake by endothelium predominated in both systems. Also, the 5HT uptake by slices was compromised to the same extent as that in the IPL after MCT or MCTP treatment in vivo, indicating that impaired 5HT uptake can be detected in the slice system, and moreover, that the magnitude of the effect is comparable to that in the IPL.

The lung slice system is a useful approach to the question of whether MCTP can injure the 5HT uptake system of the endothelial cell directly because it eliminates most blood components and other non-lung variables. Five hours was selected as an optimum preincubation time because it maximized the length of time that the slices were exposed to the monocrotaline metabolites without compromising tissue viability. 5HT uptake kinetics were similar whether or not slices were preincubated for 5 hours, and imipramine was effective in inhibiting uptake of 5HT at the end of the preincubation period. Although MCTP can polymerize and become non-reactive in aqueous solution (Mattocks, 1969; Mattocks and White, 1970), the Ehrlich reactivity present at the end of the five hour incubation period suggests that slices were exposed to MCTP for the entire incubation. It should be noted, however, that since the Ehrlich reagent reacts non-specifically with pyrrole moieties, Ehrlich reactivity does not necessarily mean that MCTP itself was present. Other pyrrolic products of MCTP metabolism or disintegration, such as RTP, could have contributed to the observed Ehrlich reactivity. In addition, some of the pyrrole measured

in the medium may have been bound to protein in the incubation medium and thus be less likely to interact with the tissue slices.

The mechanism by which MCTP injures lung parenchyma in vivo and leads to depressed uptake of 5HT and PQ is unclear. One possibility is that the MCTP directly destroys or competes for 5HT or PQ binding sites on the cell membrane. However, since lung slices preincubated with MCTP in vitro did not demonstrate decreased 5HT or PQ uptake ability, this is probably not the mechanism by which these uptake processes are affected. These findings indicate that a five hour exposure of lung tissue to MCTP was insufficient to cause functional cellular injury and suggest that MCTP-induced injury in vivo may involve more than just direct interaction of MCTP with lung tissues.

One of several indirect mechanisms may be responsible for MCTP-induced lung injury. Pyrrole metabolites of MCT can act as alkylating agents (Mattocks, 1969; Culvenor et al., 1969). Alkylation of tissue components causing interruption or alteration of cellular protein synthesis could result in membrane binding sites lowered in number and/or affinity for 5HT or PQ, thus resulting in decreased uptake of these substances. It is also possible that other factors not present in the isolated tissues mediate the damage caused by the toxin in vivo. Platelets could be involved in vivo by releasing vasoconstrictor substances or damaging endothelial cells (see Introduction). White blood cells, could be involved by releasing lysosomal enzymes or reactive oxygen species that could injure lung tissue, as has been shown in other models of lung injury (Fantone and Ward, 1982). Such interactions would require lengths of time and/or additional factors not present in a lung slice system.

Decreased 5HT uptake by an isolated, perfused lung preparation is not necessarily a result of endothelial cell injury. Since removal calculations are based on what enters and what leaves the lung, it is possible that an apparent decrease in removal could be due to shunting of perfusion medium away from injured portions of the lung. Exposing the perfusate to less endothelial surface area might be reflected as a decrease in removal. This question was resolved for the case of MCT by the lung slice experiments. After treating rats by feeding MCT, injecting MCT or injecting MCTP, the decrease in the percent removal was the same in both the IPL and the lung slices for each treatment regimen. Since shunting is not a possibility in the lung slices, these experiments support the suggestion that the decreased 5HT uptake in the IPL represents endothelial cell damage rather than hemodynamic changes.

F. In Vitro Exposure of Isolated, Perfused Lungs to MCTP

Another approach to determining whether MCTP can damage the lung directly was to mimic exposure in vivo more closely than the exposure of lung slices did. For these experiments, the lung was exposed to MCTP via the vasculature in an IPL preparation. Initially, the MCTP was added to the perfusion medium reservoir and had no discernible effects on the lung over a 90 minute perfusion. In the second experiment, the same amount of MCTP was infused directly into the pulmonary artery cannula rather than added to the reservoir. These MCTP-treated lungs developed massive edema within 30-60 minutes. The edema development was accompanied by the release of LDH into the perfusate. LDH

activity was also measured in bronchopulmonary lavage fluid, but these results are difficult to interpret. The MCTP-treated lungs had less LDH activity in the lavage fluid than the DMF-treated lungs. This may be misleading because of two factors. First, as much edema fluid as possible was drained from the airways of the MCTP-treated lungs before lavaging them. This edema fluid did contain high levels of LDH activity relative to the lavage fluid from DMF lungs. To compensate for this, the "total" LDH activity recovered from the lungs in each group was calculated. Although there was a trend toward an increase in the MCTP group, the difference was not statistically significant. Second, the development of edema in the MCTP lungs was quite rapid. Once the airways were full of fluid, excess fluid began to rise through the trachea into the ventilation system and often several milliliters of edema fluid ended up in the ventilatory trap before the perfusion was stopped. In this manner, LDH activity released by the lung may literally have been washed out into the trap and thus lost. For the same reasons, the magnitude of the increase in lavage protein concentration may be underestimated, but the fact that it is increased reflects that some damage must have occurred in the MCTP-treated lungs.

Why were the effects of MCTP on the IPL when administered via the pulmonary artery cannula different than when it was put in the perfusate reservoir? When MCTP was added to the reservoir directly the Ehrlich activity was much higher than when it was infused into the PA cannula. Added directly to the reservoir, MCTP has the opportunity to bind to the BSA in the perfusion medium, retaining its Ehrlich

reactivity but rendered less reactive toward lung tissue. When MCTP was infused into the PA cannula a larger proportion may have bound to the lung in the first pass, resulting in exposure of the lung to a higher effective dose and reducing detectable MCTP in the perfusate.

These results do not resemble what happens in vivo after a low dose of MCTP. The dose used in the first two experiments was extremely high (equivalent to 32 mg/kg in a 250 g rat). Similar doses have been shown to kill rats within 48 hours of tail vein injection (Plestina and Stoner, 1972). To establish whether the responses of the IPL in the first experiments were a function of dose, a series of isolated lungs was treated with the same dose as the intact animal would have received (the equivalent of 5 mg/kg). Again, the lungs began to develop edema about 30 minutes after MCTP administration. This still does not correspond to the situation in the intact animal. Even when the high doses were administered in vivo there was a 6-8 hour delay between injection and the first signs of edema (Plestina and Stoner, 1972; Hurley and Jago, 1975). After the 5 mg/kg dose in vivo, no increase in vascular permeability was seen until 7 days after treatment (Hamlow, Bruner and Roth, unpublished observations). Additionally, gross edema is not a primary characteristic of the response to the lower dose. Increases in wet/dry lung weight ratios are delayed and variable and, even when significant, are smaller than in experiments in vivo with high doses or in the IPL in these experiments. It is possible that the difference between the responses of the isolated lung and the lung in the intact rat are due to homeostatic mechanisms in vivo which can compensate for and/or repair MCTP-induced damage, at least for a short period. Dose may also be a

factor in the difference. Despite administration of the same nominal dose in each situation, the actual concentrations reaching the lung may differ. After injection into the tail vein, MCTP must travel to the lung, encountering numerous blood cells and a reasonable amount of vessel wall *en route*. In the IPL, the MCTP needed to travel only about 5 mm between the end of the infusion cannula and the pulmonary artery, greatly reducing the opportunity for binding to anything other than lung tissue.

The overall conclusion from the exposure of the IPL to MCTP *in vitro* is that under certain conditions MCTP can damage the lung directly. However, whether such direct damage is related to the development of MCTP-induced PH *in vivo* cannot be determined from these studies.

G. Effects of Thrombocytopenia on MCTP Toxicity

To determine whether platelets were involved in the pathogenesis of MCTP-induced PH, the effects of MCTP in thrombocytopenic rats were examined. Ideally, this experiment should have been done in rats that were thrombocytopenic for the duration of the experiment--from MCTP injection to sacrifice. Unfortunately, such prolonged thrombocytopenia has not been successfully induced in the rat. The best results reported have been a decrease to 5-10% of the normal platelet count for three days (Joris *et al.*, 1980). This regimen required 2 injections of antiserum daily and resulted in a 10% decrease in body weight by the end of the three days. Treatment for five days resulted in extremely ill animals. Longer periods of thrombocytopenia (up to 31

days) have been reported in rabbits (Moore et al., 1976; Friedman et al., 1977) but not without adverse effects on the health of the animals since there was 33% mortality in the thrombocytopenic animals in both studies. With our antiserum, platelet counts could be kept between 10 and 20% of normal for 48 hours without affecting body weight, hematocrit or general appearance of the rats. More strenuous regimens, resulting in lower platelet counts or a longer duration of thrombocytopenia compromised the health of the animals. Since thrombocytopenia for the duration of the study was not practical, we chose to make the rats thrombocytopenic at strategic intervals, using the treatment regimen which does not compromise the animals' general health. If the platelets are involved in the pathology during these periods, protection from the pulmonary hypertension and/or the accompanying lung injury might be expected.

Administering MCTP to thrombocytopenic animals (0-2 day group) did not affect the right ventricular hypertrophy or lung injury observed 14 days later. This would suggest that platelets are not involved in the initiation of the damage which is thought to occur in the first days after administration (Butler et al., 1970; Meyrick and Reid, 1982). However, the degree of thrombocytopenia induced (minimum platelet count reached was 10% of normal), may not have been sufficient to alter MCTP toxicity. The platelets remaining in the animals are presumably functional. It has been shown in models of inflammation that even when the circulating count is reduced to 0.1% of normal, platelets will still accumulate at the site of injury (Smith, M.J.H.

et al., 1976). This study also showed that reduction of the platelet count to 10% of normal did not alter the number of platelets accumulating in inflammatory exudate.

When induced at 3-5 days or 6-8 days after MCTP administration, thrombocytopenia reduced the degree of right ventricular hypertrophy but did not alter any of the indices of lung injury examined (lung weight, 5HT removal and metabolism, initial perfusion pressure, lavage LDH activity and protein concentration). The 3-5 day interval brackets the period when lung injury was first detectable after MCTP (increased lung weights, lavage LDH activity and lavage protein). The 6-8 day interval covers the period when the increases in pulmonary arterial pressure became pronounced. Protection from RVH by inducing thrombocytopenia over these periods suggests that the platelets contribute to the development of the PH at these times.

The observation that RVH could be ameliorated without a change in the magnitude of lung injury was a surprise. This could mean that the platelets contribute to the pulmonary hypertension but do not mediate the lung injury. Another possible explanation is that the 2-3 day period of thrombocytopenia merely delayed the time course of the MCTP effects by the same period. This might not appear in the indices of lung injury examined since most of these parameters reached maximum values by 7-10 days after MCTP administration (Bruner et al., 1983) and even if delayed 2 or 3 days, they could still reach a maximum value by 14 days after treatment. By contrast, RVH develops rapidly near day 14 and delaying its development by 2-3 days would probably

generate the pattern of results we observed--several animals with no hypertrophy, several with mild changes and a few with a large degree of hypertrophy. A third possibility is that repair processes (e.g., fibrosis, metaplasia) affecting the vessel wall were slowed by thrombocytopenia. As a consequence of such repair processes, the compliance of the vessels could have decreased, increasing their resistance to blood flow. Slowing the repair processes might have reduced the stimulus for RVH, decreasing the degree of RVH seen 14 days after MCTP injection.

By removing platelets from the circulation, the extent of platelet activation in response to MCTP-induced injury is presumably decreased. As illustrated in Figure 3 (p. 32), platelet activation may have a number of consequences which could contribute to the development of pulmonary hypertension. First, decreased release of agents such as 5HT and TXA_2 could reduce the vasoconstriction in the pulmonary bed and thus reduce the stimulus for right heart hypertrophy. Decreased platelet activation might also be associated with decreased release of PDGF, which could reduce the medial thickening in the pulmonary vessels if PDGF plays a role in that process. Whether the degree of medial thickening of pulmonary vessels was altered in the thrombocytopenic rats is not known.

Conceivably, PDGF could also be involved directly in the cardiac hypertrophy. Two factors argue against this possibility, however. First, there is no evidence to indicate that PDGF can stimulate cardiac muscle cell hypertrophy or hyperplasia. Although very potent, PDGF is also fairly specific, acting best on smooth muscle cells and

fibroblasts (Bowen-Pope and Ross, 1982; Huang et al., 1982). Secondly, the timing is inconsistent with this hypothesis. PDGF acts very rapidly in vitro, stimulating cell division within 24 hours and other changes as early as 4 hours after exposure (Cochran et al., 1981). The latest possible time that the platelet counts would have been reduced in our experiments was probably 9 days after MCTP. Right ventricular hypertrophy does not begin until between 10 and 14 days after MCTP treatment when the platelet counts (and hence potential PDGF levels) would be back to normal values. Decreased platelet activation in thrombocytopenic rats may also mean decreased platelet aggregation. This could result in decreased release of vasoconstrictors and PDGF and less potential for microembolization, also reducing the stimulus for the development of pulmonary hypertension.

H. Platelet Aggregation After MCTP Treatment In Vivo

To explore how platelets might be involved in the generation of MCTP-induced PH, their response to aggregating agents at various times after MCTP treatment was examined. Twenty-four hours after MCTP administration, when the platelets collected should have been exposed to MCTP, the responses to ADP, collagen and arachidonate were normal. Four days after MCTP administration, when lung injury is first detectable, the responses of platelets from treated rats were also unaltered. Seven days after MCTP administration, however, the response of platelets from treated rats to ADP was reduced. At this time, the elevation in pulmonary arterial pressure is evident in the MCTP-treated rats. Finally, 14 days after MCTP, when few if any of the circulating

platelets would have been directly exposed to MCTP, the responses to all three stimuli were depressed. Due to the design of the experiment, it is conceivable that earlier changes in collagen or AA responses may have been missed. Specifically, while three concentrations of ADP were used, producing responses ranging from near threshold ($1x10^{-6}M$) to maximal ($1x10^{-5}M$) stimulation, only one concentration each of collagen and AA were examined. The collagen concentration used produced an intermediate response. The AA concentration produced a maximal stimulus in control animals. Preliminary experiments indicated that the dose response curve to AA was extremely steep, so much so that AA appears to stimulate an all-or-none response. The threshold concentration varied widely between individual rats (50-250 μg/ml final concentration), so the maximal stimulation was chosen as the most consistent measure of the AA response.

Since the altered aggregation responses were all seen at times after MCTP such that only a small proportion of the platelets, if any, would have been exposed to MCTP, the change may not actually be in the platelets themselves. Possibly changes in some plasma components are responsible. Platelet function is extremely dependent upon the composition of the external environment of the platelet (Mustard and Packham, 1970). MCTP treatment may alter several plasma components which could affect platelet function. For example, fibrinogen and calcium are required for normal ADP aggregation (Mustard and Packham, 1970). The role of fibrinogen in this process is not clear, but human platelets have a specific receptor for fibrinogen and intraplatelet fibrinogen bridges may be important to the formation of aggregates

(Peerschke et al., 1980). There is indirect evidence that MCT may decrease plasma fibrinogen concentrations. Rats fed MCT have increased prothrombin times (Rose et al., 1945). One cause for an increased prothrombin time is hypofibrinogenemia (Duncan and Prasse, 1977). While by no means conclusive, this possibility merits further investigation. Calcium is an important regulator of platelet function although its precise role is also unclear. Intracellular calcium fluxes are necessary for the release reaction to occur (Mustard and Packham, 1970). However, extracellular calcium may inhibit the release reaction (Lages and Weiss, 1981). The effects of MCTP-induced damage on calcium are not known but might be interesting to pursue. Another plasma component which affects platelet aggregation is albumin. Addition of exogenous albumin to human PRP inhibits AA- and collagen-induced aggregation and the second phase of ADP aggregation, probably by binding AA (Silver et al., 1973). MCTP administration causes an increase in plasma protein concentrations by 14 days after treatment (Bruner, L.H., personal communication). This may reduce the platelet response. Most of the mechanistic studies discussed above were done in species other than the rat. Rat platelets differ significantly in many respects from those of other species so some caution must be used in extrapolating these observations to the rat.

MCT-induced endothelial damage may also alter PGI_2 production by the endothelium. Preliminary indications are that MCT results in increased PGI_2 production by the lung (Molteni et al., 1983). Since PGI_2 inhibits platelet aggregation, this may be a protective response if it occurs in vivo. Increased PGI_2 concentrations in the plasma are not likely to have been a factor in the platelet aggregation studies.

The PRP was allowed to rest a minimum of 45 minutes between preparation and aggregatory stimuli to allow degradation of prostaglandins and other short-lived agents with the potential to affect platelet aggregation. Another possibility for the decreased reactivity of the platelets could be a decreased number of neutrophils, and hence decreased release of PAF in the PRP of treated rats. White blood cell counts were decreased by 7 days after MCTP treatment without a change in the proportion of the various types of leukocytes (Bruner et al., 1983) suggesting a drop in the neutrophil count may have occurred although the actual decrease observed was not statistically significant. However, there is no evidence yet that rat platelets respond to PAF like the platelets of other species (Vargaftig et al., 1982).

Changes in aggregation responses were not observed 4 days after MCTP administration yet thrombocytopenia over the 3-5 day period after MCTP protected from right ventricular hypertrophy. Equally interesting was the observation that aggregation responses to ADP were decreased 7 days after MCTP and that thrombocytopenia bracketing that period also protected from right ventricular hypertrophy. Perhaps the decreased aggregation is a protective or compensatory response, developing between 4 and 7 days after MCTP administration and continuing to 14 days. If the platelets are less responsive in vivo they might be less likely to release vasoactive constituents. If so, this may retard development of PH. On the other hand, platelet aggregation may not be involved in MCTP toxicity in vivo at all. For example, the major contribution of the platelets to MCTP toxicity may be PDGF.

Altered aggregation activity by 7 days after administration may not affect the quantity of PDGF released at crucial times for the stimulation of medial thickening in the vessels.

I. Effects of MCTP Treatment on Fawn-hooded Rats

The platelet defect in the fawn-hooded rats is a limited one in that all platelet functions do not seem to be compromised. Although ability to take up and release 5HT and adenine nucleotides is decreased (Raymond and Dodds, 1975) and the dose response curve for aggregation to collagen and arachidonic acid is shifted to the right (Wey et al., 1982), FH rat platelets do respond normally to ADP (Raymond and Dodds, 1975) and produce the same amount of TXB_2 as Sprague-Dawley rat platelets (Wey et al., 1982). There is indirect evidence that the FH rats have normal PDGF function as well. PDGF is believed to be responsible for the intimal proliferation following endothelial cell injury in models of atherosclerosis (Moore et al., 1976; Harker et al., 1976). Fawn-hooded and Sprague-Dawley rats show the same degree of proliferative response after aortic de-endothelialization, suggesting that both strains can support a mitogenic response (Tiell et al., 1982). While not a platelet defect, FH rats do have one other vascular change--aortic rings from FH rats produce less 6-keto-$PGF_{1\alpha}$ than do rings from SD rats, suggesting that the endothelium produces less PGI_2 (Wey et al., 1982).

Since the FH rats exhibited no protection from MCTP-induced pulmonary hypertension, the ability of the platelets to release and/or accumulate 5HT and ADP may not be essential to the pathogenesis. The

platelets could still be involved by releasing PDGF or through the production of TXA_2 since these are both unchanged in the FH rats. The decreased PGI_2 production might even be enhancing the susceptibility of FH rats to pulmonary hypertension by removing a potential protective influence. Prostacyclin may protect from PH *in vivo* by inhibiting platelet aggregation and promoting vasodilation.

The removal and metabolism of perfused 5HT was greater in IPL of FH rats than of SD rats. Perhaps the higher removal is an adaptive response. Platelets remove 5HT from the plasma (Pletscher, 1968). Platelet uptake and pulmonary removal of 5HT in plasma may protect the arterial circulation from inappropriate effects of the amine. Since platelets from FH rats have a decreased ability to take up 5HT, enhancement of the carrier mechanism in the endothelial cells may compensate to keep 5HT concentrations low. The higher 5HT metabolite production by lung could have two origins. It may simply reflect the increased uptake or it may result from increased monoamine oxidase activity in the endothelial cells.

J. Vascular Reactivity in Isolated, Perfused Lungs After MCTP Treatment

A number of factors, alone or combination, could be responsible for the increases in pulmonary vascular reactivity observed after MCTP treatment. It is probable that a large component is the result of structural changes (i.e., hypertrophy/hyperplasia) in the pulmonary blood vessels. Folkow and colleagues (1973) have described a model in which increases in vessel wall/lumen ratio will cause the vessel to

respond with a proportionately greater increase in resistance with the same degree of smooth muscle activity as would be seen in a vessel with a smaller wall/lumen ratio. Structural remodelling has occurred by both 7 and 14 days after MCT treatment earlier as evidenced by morphological evaluation of vessel wall thickening (Ghodsi and Will, 1981; Kay et al., 1982). The existence of structural changes after MCTP is suggested by the elevation of baseline perfusion pressure in isolated lungs of the rats treated with MCTP 14 days earlier. These structural changes may be largely responsible for the increased vascular reactivity to 5HT and AII, at least at 14 days after treatment.

Another possible component of the enhanced vascular response is a change in the responsiveness of smooth muscle cells to stimulation. Changes in the membrane potential of smooth muscle cells of the main pulmonary artery (depolarization) and small muscular pulmonary arteries (hyperpolarization) have been described in rats with MCT-induced pulmonary hypertension (Suzuki and Twarog, 1982). Small pulmonary artery smooth muscle cells from MCT-treated rats, in spite of their hyperpolarized state, responded to Na^+-deficient solutions with a larger and longer depolarization and contraction than did comparable arteries in control rats. Such membrane changes may facilitate contraction in the intact vessel, leading to an enhanced response. It is also possible that a change occurs in the vascular receptors for vasoactive compounds (e.g., increased numbers, altered affinity or accessibility) or in the coupling of receptor occupancy to excitation or of excitation to contraction. Such changes have been proposed and/or examined for models of systemic hypertension (Finch

and Haeusler, 1974; Lais and Brody, 1975) but not for chronic pulmonary hypertension. Changes in the responsiveness of the smooth muscle cells may be more important than structural changes to the enhanced vascular responsiveness seen 7 days after MCTP treatment since there was no increase in baseline perfusion pressure of the isolated lungs at this time.

When 5HT challenge was preceded by AII, the response to 5HT was greater than when 5HT alone was administered, suggesting an interaction between the two compounds in the lung preparation. The AII concentration in the perfusate was probably small when the 5HT was introduced, because perfusion pressure had returned to baseline several minutes before the 5HT challenge. 5-Hydroxytryptamine is known to potentiate the contractile response to many agents, including AII (Van Nueten et al., 1982). Accordingly, part of the vasoconstrictor response to 5HT following AII challenge may have been due to synergism between 5HT and residual AII in the perfusion medium.

Fourteen days after treatment the lungs from pulmonary hypertensive rats had increased vascular reactivity to both AII and 5HT. Although the 5HT responses were lower when not preceded by AII administration, the MCTP-treated lungs still had a larger response than the DMF lungs. Thus, 14 days after MCTP treatment, the vascular response to 5HT may be enhanced by but does not require the presence of AII.

A role for serotonin in the etiology of certain types of primary pulmonary hypertension has been proposed. The anorectic drug aminorex causes pulmonary hypertension in rats (Lullmann et al., 1972; Mielke et al., 1973), and its consumption was associated with a surge of

cases of primary pulmonary hypertension in Europe in 1967/68 (Follath, 1971). In rats, the pulmonary hypertension resulting from aminorex administration is associated with decreased uptake of 5HT from the vasculature and decreased inactivation of 5HT via inhibition of monoamine oxidase in the lung (Mielke et al., 1973; Seiler et al., 1974). MCT also decreases the uptake of 5HT by the pulmonary vasculature (Gillis et al., 1978; Huxtable et al., 1978; Roth et al., 1981; Hilliker et al., 1982), but does not affect the monoamine oxidase activity of lung tissue (Gillis et al., 1978). Nonetheless, MCT or aminorex, by impairing pulmonary removal of blood-borne 5HT, could favor increased concentrations of 5HT in the pulmonary vasculature which could lead to vasoconstriction. Imipramine inhibits 5HT uptake, so if the decrease in 5HT removal by lungs of MCTP-treated rats were responsible for the increased reactivity, then the pressor response in imipramine-treated lungs might also have been expected to increase. The addition of imipramine to the perfusion medium in these experiments did not enhance the response to either 5HT or AII, indicating that decreased removal of 5HT by the pulmonary vasculature is not responsible for the acute hyperreactivity. This is consistent with the findings of Rickaby et al. (1980), who showed that in the dog lung the primary site of vasoconstriction is the arterial side of the circulation and that imipramine did not alter the vasoconstriction. The role of decreased 5HT removal in the development of chronic pulmonary hypertension by MCTP remains to be determined.

In contrast to MCTP-induced pulmonary hypertension, the pulmonary hypertension resulting from chronic alveolar hypoxia does not cause an

increased response to 25-75 μg 5HT, although increased responses to 0.25-0.75 μg AII occur (Greenlees et al., 1982). Many of the morphological changes in the pulmonary vasculature are similar in pulmonary hypertension induced by chronic hypoxia and by MCT (or MCTP); that is, both involve muscle extension into previously non-muscular arterioles, medial thickening, and narrowing of peripheral arterioles (Meyrick et al., 1980; Rabinovitch et al., 1979). A major difference between the two models lies in the reversibility of the lesions. Removal of the hypoxic stimulus allows the reduction or return to normal values of arterial thickening, right ventricular hypertrophy, and polycythemia (Hislop and Reid, 1977; Kay, 1980) in rats with hypoxic pulmonary hypertension. Monocrotaline-induced pulmonary hypertension is by contrast a progressive disorder which usually results in death of the animal. The differences between the two models are likely due to differences in the mechanisms by which hypoxia and MCT/MCTP act. Monocrotaline probably causes an early chemical injury to the pulmonary vessels, possibly to the endothelial cells (Butler et al., 1970; Meyrick and Reid, 1982). The primary event in pulmonary hypertension from chronic hypoxia is considered to be vasoconstriction induced by alveolar hypoxia (Wagenvoordt and Wagenvoordt, 1977). Whether the difference in vascular response to 5HT in MCTP-treated rats is the result of the injury sustained by the lung after MCTP exposure or arises from a response to the vascular changes (e.g., arterial medial thickening) common to both MCTP and chronic hypoxic pulmonary hypertension is unclear.

The vascular hyperreactivity is more pronounced 14 days following MCTP treatment than at 7 days. This suggests that the enhancement in vascular reactivity develops along with the pulmonary hypertension. While it seems likely that the increased response to vasoconstrictors plays a role in the maintenance or progression of the pulmonary hypertension, further study is needed to determine whether the hyperreactivity is a cause or a consequence of monocrotaline-induced pulmonary hypertension.

K. Effects of Drug Treatments on MCTP Toxicity

Of the drugs tested, three (HD, DX, SP) afforded some degree of protection from MCTP-induced right ventricular hypertrophy. One mechanism of action was common to all three--the ability to inhibit prostaglandin synthesis. Hydralazine and sulfinpyrazone tend to inhibit platelet TXA_2 synthesis more than vascular PGI_2 production (Greenwald et al., 1978; Srivastava and Awasthi, 1982; Livio et al., 1980), while dexamethasone reduces all prostaglandin synthesis by decreasing the availability of substrate (Flower, 1978). This common mechanism would suggest that platelet thromboxane synthesis could be important in the development of MCT-induced pulmonary hypertension. Other actions of these drugs could have contributed to the protection, too. Hydralazine, as a potent vasodilator, may have reduced the pulmonary vascular resistance, thereby reducing the stimulus for right heart hypertrophy. Kay and coworkers (1976) found that chronic cinnarizine administration did not protect from MCT toxicity. Cinnarizine inhibits calcium fluxes into depolarized smooth muscle cells, thereby reducing smooth muscle contraction to a wide variety of stimuli

(Godfraind and Kaba, 1969). Reduction of vasoconstriction may not be enough, then, to protect from RVH. However, there was no indication in this study of whether the dose of cinnarizine used actually decreased vasoconstriction *in vivo*, so the vasodilatory action of HD is not completely ruled out as a possible mechanism.

Dexamethasone also has other actions beside the inhibition of prostaglandin synthesis. As an anti-inflammatory and immunosuppressive agent it could have reduced the inflammatory response in the lung and thus reduced the injury. However, dexamethasone's protective effect may be unrelated to its pharmacological actions. The DX treatment regimen used in this study caused a significant drop in the weight gained by both the DX/DMF and DX/MCTP groups over the experimental period. As will be discussed below, animals whose weight gain and/or growth is restricted are protected from the RVH caused by MCT (Hayashi *et al.*, 1979) or MCTP (Ganey and Roth, 1983). Accordingly, the DX protection may have been the result of its effects on growth rather than a specific pharmacological effect.

Two of the drugs used in these studies did not protect from MCTP effects. Acetylsalicylic acid, despite its action as a prostaglandin cyclooxygenase inhibitor, did not alter the lung damage and RVH in response to MCTP. The dosage regimen used may be reason for the lack of effect. In attempting to inhibit the platelets selectively without a prolonged effect on vessel wall prostaglandin synthesis there may have been periods at the end of the dosing interval when even platelet prostaglandin synthesis was near normal. Without maintenance of a minimum effective dose throughout the experimental period, ASA might not exert a protective effect.

The other drug tested which did not protect from MCTP toxicity was metergoline. At the dose used (1 mg/kg, daily), MTG has selective effects on 5HT systems in the central nervous system (Sastry and Phillis, 1977) but its effects on 5HT receptors in the peripheral vasculature are not known. Many 5HT antagonists are also partial agonists (e.g., imipramine, Rickaby et al., 1980). If MTG acted as a partial agonist in the pulmonary vasculature, that might explain its lack of protective effect. Other investigators have explored the potential role of 5HT in MCT-induced pulmonary hypertension by pretreating rats with p-chlorophenylalanine (PCPA), an inhibitor of 5HT synthesis. Rats treated with PCPA have a decreased lung 5HT content, less of an increase in pulmonary arterial pressure (Carillo and Aviado, 1969) and reduced right ventricular hypertrophy (Tucker et al., 1982) after MCT administration compared to rats treated only with MCT. This could mean that 5HT is important in the development of MCT-induced pulmonary hypertension, but PCPA might also have acted by interfering with hepatic bioactivation of MCT. We plan to examine the effects of ketanserin, a 5HT receptor inhibitor that apparently acts as a pure $5HT_2$ receptor antagonist in the vasculature (Van Nueten et al., 1981), on MCTP-induced pulmonary hypertension.

In addition to the drugs discussed above, other drugs or manipulations have also been used in attempts to alter MCT toxicity. Restricting the food intake of MCT-treated rats so that the animals maintained but did not gain body weight reduced the vascular injury, right ventricular enlargement and lung weight increases which occurred in control animals fed an unrestricted diet (Hayashi et al., 1979).

This protective effect was probably not due to a decreased conversion of MCT to MCTP by the liver since similar results have been observed after the administration of MCTP (Ganey and Roth, 1982). The effects of diet restriction on the lung have not been well-characterized. One explanation for the protective effect might be that diet restriction caused immunosuppression. Inflammatory responses are common in MCT-treated animals and may play a part in initiating or promoting the pulmonary damage. A protein-free diet decreases the inflammatory response to viral infection in the mouse lung (Jakab et al., 1981). Malnutrition in general also causes immunosuppression, primarily by actions on mechanisms of cell-mediated immunity (Gross and Newberne, 1980).

Pretreatment with 6-hydroxydopamine (6-OHDA) has been shown to decrease the RVH and to reduce marginally the pulmonary vascular hypertrophy in rats given a single injection of MCT (Tucker et al., 1982). These authors suggested that this protection was due to decreased sympathetic nervous system activity. However, 6-OHDA when injected intracerebroventricularly causes decreased food intake (Kostrzewa and Jacobowitz, 1974). 6-OHDA did not prevent the drop in body weight gain typically seen after MCT treatment, so in this case, the drug may have protected via its effects on food intake.

Studies with prednisolone, an immunosuppressant drug, do not strongly support the hypothesis that an immune mechanism is involved in MCT-induced PH. Chronic treatment with prednisolone after a single injection of MCT caused a slight decrease in systolic right ventricular pressure 21 days after MCT but exhibited no effect on this

parameter 28 days after MCT (Tanabe et al., 1981). Right ventricular hypertrophy was not altered at either time. The mortality was somewhat less in the group of rats receiving both MCT and prednisolone (41% versus 59% in the MCT only group). The rationale for the dose of prednisolone administered was not presented nor were any measures of its efficacy as an immunosuppressant at this dose reported. It may be that the dose used was only marginally effective and that would explain the relative lack of protection. On the other hand, the immune system may not be important in the development of MCT-induced PH. Further study is warranted to clarify this point.

Propranolol is another agent which will protect from MCT-induced PH. Administration of propranolol in the drinking water concomitantly with MCT for 28 days prevented the RVH observed after administering MCT along in the drinking water for the same period (Huxtable et al., 1977). The protective effect might simply reflect decreased intake of MCT (e.g., if propranolol taste deterred drinking) or impaired activation of MCT to MCTP. The water intake and thus cumulative MCT dose was not reported. However, a recent report (Russell et al., 1982) showed that propranolol decreased the pulmonary hypertension resulting from chronic hypoxia in pigs, suggesting that propranolol may have a pharmacological action which is effective against pulmonary hypertension. Classically, propranolol is considered a beta-adrenergic antagonist (Nickerson and Collier, 1975). It also has membrane stabilizing effects comparable to those of local anesthetics, resulting in a decrease in heart rate and contractility and in the conduction velocity. Renin secretion by the kidney in response to sympathetic

stimulation is decreased, which could ultimately reduce the plasma concentration of AII (Nickerson and Collier, 1975). Propranolol also decreases 5HT uptake by platelets (Rudnick et al., 1981) platelet aggregation (Nathan et al., 1977) and inhibits phospholipase A_2 (Vanderhoek and Feinstein, 1979). One or more of these actions could be involved in the protective effects of propranolol in pulmonary hypertension models.

Another drug which reduces AII concentrations and protects from MCT-induced PH is captopril, an angiotensin converting enzyme inhibitor. Rats fed MCT and captopril in their drinking water developed less RVH than rats fed MCT only (Molteni et al., 1982). Captopril has also been shown to decrease pulmonary vascular resistance in patients with primary pulmonary hypertension (Niarchos et al., 1982). These results suggest that AII-induced vasoconstriction could be involved in the development and/or maintenance of pulmonary hypertension.

Zoxazolamine is a spinal cord depressant which inhibits skeletal muscle contraction (Burns et al., 1958). It also induces and is metabolized by hepatic microsomal enzymes (Conney, 1967). Chronic administration of zoxazolamine in the drinking water after a single injection of MCT prevented pulmonary MCT effects in 6 out of 15 treated animals (Kay et al., 1976). Seven of the fifteen were not protected at all. One rat had RVH 120 days after the MCT injection but no evidence of pulmonary change morphologically. Zoxazolamine may have acted by altering the metabolism of MCT to MCTP. In the animals which survived, detoxifying pathways may have been induced in the liver.

Based on the drugs which ameliorated MCT- or MCTP-induced pulmonary hypertension, reduction of the concentration of available vasoconstrictors would seem to be the most successful mechanism. At least three vasoconstrictors are implicated: prostaglandins (especially TXA_2), 5HT, and AII. Further work on the potential roles played by these compounds seems merited.

CONCLUSIONS

This project was conceived after consideration of three major facts. First, MCT administration caused pulmonary hypertension. Second, MCT required metabolic activation to a pyrrole moiety to cause pulmonary hypertension. Third, the lung did not seem to convert MCT to a pyrrole form. It had previously been suggested that the liver activated MCT and released the metabolite(s) into the bloodstream. This reactive metabolite(s) could then damage the lung, the next organ encountered in the circulation (Barnes *et al.*, 1964; Butler *et al.*, 1970). We began our investigations of the mechanism by which MCT produces pulmonary hypertension by examining whether the reactive metabolites could initiate lung injury directly in systems *in vitro*.

A pyrrole metabolite of MCT (MCTP) is capable of initiating damage by direct interaction with the pulmonary vasculature. This was demonstrated by studies in the isolated, perfused lung wherein MCTP infusion caused edema and cellular damage. Certain aspects of lung injury are slower to develop, however. In lung slices, for example, short-term damage to the 5HT and PQ uptake systems was not observed after exposure *in vitro* to MCT metabolites. This correlates well with observations that changes in 5HT uptake by IPL are delayed several days after MCT or MCTP administration *in vivo*. Overall, these results are consistent with the suggestion of Meyrick and Reid (1982) that

MCT-induced pulmonary hypertension has two phases--one of initial damage which triggers events that result in a later stage of vascular injury and development of pulmonary hypertension.

We also considered ways in which the metabolite(s) released by the liver could cause pulmonary damage indirectly via interaction with a component of the blood which could in turn injure the lung. Because platelet-containing thrombi were a feature of MCT-induced lung pathology and MCT treatment caused a large decrease in the circulating platelet count we chose to study the role of the platelet. This initial hypothesis that the platelet was damaged by MCTP in the blood and thence injured the lung was not borne out by experimentation. Induction of thrombocytopenia for the first two days after MCTP treatment (when MCTP concentrations would be highest) did not alter the right ventricular hypertrophy or lung damage 14 days after MCTP treatment. However, reduction of the platelet count at later times during the experimental period did reduce the degree of right ventricular hypertrophy, indicating that the platelet is involved in the development of the pulmonary hypertension. Furthermore, responses of PRP to platelet aggregating agents were depressed at later times after MCTP treatment.

As illustrated in Figure 3 (p. 32), there are numerous ways in which the platelet could be involved in the pathogenesis of MCT-induced pulmonary hypertension. Examination of the possibility of serotonergic mechanism was attractive since 5HT is released from platelets after a variety of stimuli and might be involved in the pulmonary hypertension by virtue of its vasoconstrictor properties.

Isolated lungs from MCTP-treated rats showed increased pressor responses to 5HT compared to DMF-treated lungs. This was not a specific effect of MCTP on the serotonergic system, however, since pressor responses to AII were also increased. MCT and MCTP decrease the ability of the lung to remove circulating 5HT, which might cause increased intrapulmonary 5HT concentrations in the blood, but this probably does not contribute directly to the increased pulmonary arterial pressure. Most of the 5HT removal takes place in the capillary bed (Rickaby et al., 1980) so resultant increases in 5HT concentration would occur in the venous side of the circulation, downstream from the resistance vessels of the pulmonary circulation (Rickaby et al., 1980). Increased venous concentrations of 5HT might cause capillary permeability and edema though, increasing pulmonary vascular resistance by that mechanism.

Little conclusive evidence for a specific role for 5HT in the etiology of MCT-induced pulmonary hypertension was established. Platelet uptake and release of 5HT probably do not play a major role since fawn-hooded rats, a strain with a congenital defect in these functions, showed no protection from MCTP-induced pulmonary hypertension. Previous studies showed that PCPA reduces MCT-induced pulmonary changes (Carillo and Aviado, 1969; Tucker et al., 1982), suggesting a role for 5HT, but our attempts to protect rats from MCTP-induced pulmonary hypertension by treatment with metergoline were unsuccessful. Further work is warranted to clarify the role played by 5HT in this model.

Platelets could be acting by a non-serotonergic mechanism. All of the drug treatments which were effective in reducing the right ventricular hypertrophy caused by MCTP inhibit the synthesis of prostaglandins, particularly TXA_2, by the platelets. As a vasoconstrictor and stimulator of platelet aggregation, TXA_2 may contribute to the pulmonary hypertension. Another non-serotonergic mechanism of potential importance is the release of PDGF by the platelets. By stimulating structural remodelling of the pulmonary vasculature, PDGF could be involved in the development of the hypertension. The roles of prostaglandin synthesis and PDGF release by the platelet need to be explored further.

In summary, MCTP would seem to act by causing initial damage to the endothelial cell, probably by covalent binding, although the immediate consequences and form of this initial injury are not known. Some time later, platelets, vasoconstriction and medial thickening contribute to the development and/or maintenance of the MCTP-initiated changes. Ultimately, pulmonary arterial pressure is increased, stimulating hypertrophy of the right ventricle. This project identified some of the factors and mechanisms which are involved in the transduction of the initial MCTP injury into pulmonary hypertension. Much work remains to be done before the pathogenesis is fully understood. Since MCT-induced pulmonary hypertension is similar in so many respects to human primary pulmonary hypertension, it is hoped that information about the mechanisms involved in the MCT model can be applied to improving diagnosis, therapy and/or prognosis of the human disease.

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