STUDIES ON THE EFFECT OF PARAQUAT ON THE RAT LUNG

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ABSTRACT

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By

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Paraquat (Methyl Viologen) is the generic name of the compound, 1,1'-dimethyl 4,4', dipyridylium. It is a broad spectrum herbicide effective against broad leaf weeds, grasses and aquatic weeds, and it is rapidly deactivated on contact with the clay in soil.

On account of its advantages over other herbicides, it has been increasingly used all over the world. Medical interest in paraquat has arisen as a result of the peculiar and often fatal pulmonary damage in the mammalian species which may occur after ingestion of small quantities. It has caused over 200 deaths world-wide since its introduction in 1962. Death following paraquat poisoning is usually the result of progressive fibrosis and epithelial proliferation in the lungs. Thus, paraquat poisoning usually causes a progressive respiratory disease, unresponsive to any therapy, resulting in death.

Rational therapeutic measures have not been developed because of lack of information on the precise mechanism of action and optimal methods for removal of the poison. Thus, the overall purpose of this study was to determine the effect of other chemical agents on the toxic effects of paraquat and how these could be adopted for use in the management of paraquat poisoning.

The objectives of this project were three fold. The first was to study the effect of paraquat on the isolated rat lung preparation and how its effects may be modified by other chemical agents. The agents used were those that had been reported to offer some potential for protecting against or increasing paraquat toxicity and might provide insight into the mechanism of paraquat poisoning. The second objective was to study the modification of the effect of paraquat toxicity in vivo in the presence of other chemical agents in both acute and chronic experiments and how these may elucidate the mechanism of paraquat toxicity. The third objective was to investigate how the paraquat affected lung handled endogenous substances which are normally activated or metabolized by the lung and how their control may affect the outcome of paraquat poisoning.

The experimental data presented in this dissertation show that perfusing the isolated rat lung with paraquat resulted in the rapid production of marked edema, measured by the percent change in the weight of the lung (mean 37.7 $\pm$ 17.7 SEM).

It was also observed that perfusing the rat lung with paraquat in the presence of mannitol or propranolol significantly reduced the amount of edema formed.

This observation suggested that propranolol and mannitol may afford some protection against paraquat poisoning, but this observation was not confirmed in the in vivo experiments with propranolol.

The concentration of the cyclic nucleotide, cyclic AMP, in the lung was increased significantly after perfusing with paraquat and this increase was reduced in the presence of propranolol.

In both the acute and chronic in vivo experiments, significant increases were observed in lung cyclic AMP concentrations in rats treated with paraquat. In the chronic experiments in which groups of rats received paraquat, paraquat and propranolol, or paraquat and theophylline, the highest concentrations of cyclic AMP and cyclic GMP were found in the group of rats which received paraquat and theophylline, and the percentage mortality was highest in this group but not significantly different from the other groups which received paraquat. The mean concentrations of paraquat in the lungs of the different groups were not significantly different. Cyclic AMP and cyclic GMP concentrations in other organs including the liver, spleen, kidney and thymus, which are known to be affected by paraquat, unlike the lung, were not significantly different from controls. It was also observed that paraquat induced cyclic nucleotide changes in the rat lung were not endogenous catecholamine dependent since levels in resperine treated rats were not significantly different from non-reserpinised paraquat treated rats.

The isolated lung from the paraquat-treated rats perfused with ^{3}H-PGE_2 showed significant inhibition of PGE_2 metabolism. This was probably due to an impairment of the uptake mechanism for PGE_2 since significantly more unchanged PGE_2 was present in the venous effluent from the lungs of treated rats and the levels of the metabolites (15-keto-PGE_2 and 13,14-dihydro-15-keto-PGE_2) in the lung homogenates of the treated and control groups were not significantly different. Interference with the pulmonary mechanism for inactivating endogenous vasoactive hormones such as PGs by drugs, toxic chemicals, atmospheric pollutants or disease is probably more important than

hitherto appreciated. In paraquat poisoning, interference with excretor as a result of circulating abnormal levels of renal vasoconstrictor hormones such as $PGF_{2\alpha}$, would accentuate toxic effects particularly on the target organ, the lung.

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By

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A THESIS

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Michigan State University
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DEDICATION

This work is dedicated to my wife Saah, and Koshie Odarley, Odarkor, Odartey and Odartei, our delightful children.

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I would like to express my sincere appreciation to the graduate committee members: Drs. Theodore M. Brody, Robert Echt, James E. Gibson, and Andrew M. Michelakis, for their assistance in the preparation of this thesis. I would especially like to acknowledge Dr. Andrew M. Michelakis for his guidance, constructive criticism and encouragement during this investigation and throughout my graduate studies. I am indebted to Dr. Robert Echt for his constant interest and invaluable help. I gratefully acknowledge the technical assistance of Mr. Paul Susan and Ms. Ivy Mao, and the typing skills of Ms. Sharon Hallabrin.

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INTRODUCTION

Paraquat (methyl viologen) is the generic name for 1,1'-dimethyl-4,4'-dipyridylium. It is available either as the dichloride or dimethyl sulphate salt, both of which are water soluble. Paraquat is a broad spectrum herbicide effective against broad leaf weeks, grasses and aquatic weeds. Paraquat has been reported to be firmly bound when it contacts most types of soils and upon adsorption can be degraded by several micro-organisms present in the soil. Application to crops results in a low level of contamination.

The following formulations are available in the United States:

1.	ORTHO Paraquat Cl	29.1% solution (21.7 weight-% cation)
2.	ORTHO Dual Paraquat	42% Solution (21.7 weight-% cation)
3.	Chevron Industrial Weed and Grass Killer	21.07 weight-% cation
4.	ORTHO Spot Weed and Grass Killer	0.44% solution (0.2 weight-% cation)

ORTHO Spot Weed and Grass Killer is unlikely to be a problem in accidental poisoning because of its low paraquat content and the fact that it is only marketed in pressurized cans which dispense a foam. Outside the U.S. paraquat is marketed as 20% concentrate, Gramoxone, and in a 5% granular form, Weedol.

Medical interest in paraquat has arisen as a result of the peculiar and often fatal pulmonary toxicity in mammals which may occur after ingestion of small quantities. It is estimated to have caused over two hundred human deaths world wide since its introduction in 1962. Most of these deaths have followed accidental oral ingestion. The fatal dose of paraquat in adult man is thought to be about 15ml of a 20% solution, or a "mouthful" as described in most case histories (Kimbrough, 1974). Death following paraquat poisoning is usually the result of progressive fibrosis and epithelial proliferation. This effect has been described in both humans and animals, although there is a marked difference in the oral acute lethal doses observed between species (Clark et al., 1966; Murray and Gibson, 1972).

Rational therapeutic measures have not been developed for paraquat poisoning because of the lack of information on the precise mechanisms of action, and optimal methods for the removal of the poison.

Human Toxicity of Paraquat

Serious paraquat poisoning in humans has occurred only after ingestion or parenteral administration (Almog and Tal, 1967). Exposure of the skin to a solution of paraquat produces erythema and reactive hyperkeratosis which may be associated with pustule formation. There is ulceration and necrosis two to three days after short periods of contact with the mucous membrane and conjunctiva of the eye (Conning et al., 1969).

Signs and Symptoms

In cases of paraquat ingestion, there is usually an immediate burning discomfort of the mouth and pharynx, followed by ulceration due to the severe irritating effect of paraquat. This is generally followed by repeated vomiting. If the dose ingested was large (i.e., 6-8 oz.), the lungs, kidneys, liver, and adrenals may be severely affected initially followed by possible fatal pulmonary edema within 24 to 72 hours. When smaller amounts are taken there may be oliguria, increase in blood urea nitrogen and albuminuria as a result of acute renal failure. Jaundice is also sometimes noted. The initial phase is followed by a latent period sometimes lasting as long as two weeks during which time the patient feels well and kidney function generally improves.

These symptoms are superceded by predominantly pulmonary signs and symptoms including increasing dyspnoea, cyanosis, and pulmonary congestion. Pathologic changes include necrosis of alveolar epithelial cells and accumulation of alveolar macrophages (Witchi and Kacew, 1974). Pulmonary infiltrates are detected on chest roentgenograms, and physiologic studies reveal hypoxemia, decreased lung volumes, low lung compliance, and impaired diffusing capacity for carbon monoxide (DLCO) (Gardner, 1972; Matthew et al., 1968). Later stages are characterized by the development of pulmonary fibrosis and eventual death from respiratory failure.

Lung pathology in paraquat poisoning has been studied extensively. Thurlbeck and Thurlbeck (1976), noted a considerable topographic variation in the severity and nature of lung lesions. They described two distinct forms of fibrosis. In one form, there was

marked intra-alveolar edema in much of the lung and active fibroblastic proliferation of the lung interstitium. Hyaline membranes lined some of the air spaces and honey-combing with disorganization of the alveolar pattern of the lung was evident. Also, distinctive areas of the lung structure were completely disorganized and replaced by many small (0.05mm-2.0mm) cysts lined by fibrous tissue. This type of lesion is similar to fibrosing alveolitis in man.

A second type of fibrosis is characterized by preservation of the framework of the lining architecture with the formation of abundant loose fibrous tissue within the alveolar spaces. Thurlbeck and Thurlbeck (1976) suggested this may represent the organization of protein-rich edema fluid which pours into alveoli at an early stage of paraquat poisoning. On the contrary, Smith and Heath (1974), claimed that the formation of a fluid exudate during the early stages of paraquat poisoning is not necessary for the development of pulmonary fibrosis and that paraquat or its metabolites, directly stimulates an infiltration of profibroblasts into the lung.

Oxygen poisoning shares with paraquat the special sensitivity of type I epithelial-cells to damage but the early phase of paraquat poisoning is different from early oxygen poisoning (and ozone and radiation), where damage to endothelial cells plays a prominent role. The fibrotic and proliferative lesions described, as well as marked edema are very similar to the lesions described in oxygen poisoning. Although some of the patients studied were treated with oxygen terminally, Thurlbeck and Thurlbeck (1976), clearly identified the lung lesions as not caused by oxygen therapeutically administered since clinical pulmonary involvement occurred before oxygen was

administered and also occurs in animals to which oxygen has not been given. However, some features of oxygen poisoning, notably severe edema may have been an added feature to the underlying paraquat lesion. The similarity to oxygen poisoning and the observation that hyperoxic environment increased the lethality of paraquat in rats (Fisher et al., 1973) has led to the suggestion that paraquat may particularly affect the lung because of its high ambient oxygen.

Although death due to paraquat poisoning is usually due to progressive respiratory failure (Malone et al., 1971; Matthew et al., 1968; Gardner, 1972), fatalities have occurred less frequently from uremia or from cardiac involvement (Oreopulous et al., 1968; Gardner, 1972).

In most cases of paraquat poisoning in man, death occurred approximately one to four weeks after ingestion.

Therapeutic Approach to Paraquat Poisoning

The therapeutic measures used thus far to manage paraquat poisoning have been unsuccessful. The therapeutic approach has been based on three general principles:

a. Prevention of paraquat absorption
b. Rapid excretion of absorbed paraquat
c. Modification of the tissue effects of absorbed, non-excreted paraquat.

Prevention of paraquat absorption--Gastric lavage and administration of cathartics are well established general measures for the treatment of some cases of poisoning including paraquat poisoning. Many adsorbents have been shown to be effective against absorption of

paraquat in vivo, but only bentonite and Fuller's earth have been effective in vivo (Clark, 1975; Staiff, et al., 1973).

The effectiveness of these agents is probably due to their prevention of gastro-intestinal absorption of paraquat. Smith et al. (1974), have demonstrated an active process for the uptake of paraquat into the lungs in both human and rat lung slices and it is, thus, important that specific measures to inhibit paraquat absorption should be considered an important part of the management of paraquat poisoning.

Rapid Excretion of Absorbed Paraquat

Increased urinary excretion of paraquat following forced diuresis with saline solution and mannitol has been documented (Fisher et al., Kerr et al., 1968). Forced diuresis is apparently a more effective means of removing absorbed paraquat than peritoneal dialysis (Fisher et al., 1971). Since paraquat has been detected in the urine for as long as 31 days after ingestion (Beebeejaum et al., 1971), continued, as well as early efforts to eliminate absorbed paraquat may be lifesaving. Hemodialysis used early is effective in reducing the plasma levels of absorbed paraquat (Grundies et al., 1971).

Modification of Tissue Effects of Absorbed Paraquat

Paraquat toxicity in rats is greatly enhanced by oxygen. Hyperoxic environment (100% inspired oxygen) markedly accelerates the mortality of rats given lethal doses of paraquat (Fisher et al., 1973), on the other hand, Smith and Rose (1977), have shown that paraquat poisoned rats placed immediately after dosing in atmosphere containing 10% oxygen, paradoxically died sooner than those left in air and there was no reduction in overall mortality. This is claimed

to be due to an increased rate of accumulation of paraquat by the lung perhaps resulting from an increased perfusion of the lung by blood (Smith and Rose, 1977).

Other Regimens

Some reports have described treatment of patients who have ingested paraquat with corticosteroids (Malone et al., 1971; Duffy et al., 1968; Lathwaite, 1975), immunosuppressive agents (Malone et al., 1971), or the antifibrotic agent, potassium aminobenzoate (Laithwaite, 1975). However, in the majority of cases, suppressive agents have not been effective. Other therapeutic measures available include the use of d-propranolol and superoxide dismutase (SOD). A regimen currently in use at the Royal Postgraduate School, Hammersmith, London, involves a stomach washout as soon as possible after ingestion followed by the administration of a 30% suspension of Fuller's earth (200-300 ml), along with a cathartic. This is continued for several days in order to prevent the absorption of paraquat. In cases of serious paraquat poisoning where there is impairment of renal function, hemodialysis is used. Oxygen administration is avoided as much as possible. Along with the above measures, SOD is administered both intravenously and by inhalation for a period of at least one week following the incident. In addition, d-propranolol is given intravenously in an attempt to reduce the uptake of paraquat into the lung. Beclomethasone, a synthetic corticosteroid preparation, is administered from pressurized aerosol cannisters to limit the inflammatory process in the lung. The use of oxygen in paraquat poisoning has become a controversial issue. There are theoretical reasons and evidence from

animal studies which contraindicate the use of oxygen. There is in addition, recent evidence which contraindicates the use of low oxygen therapy in cases of human poisoning unless it can be shown that the paraquat concentration in circulating blood is extremely low (below 0.05 nmoles/ml) such that enhanced lung accumulation will not occur (Smith and Rose, 1977).

Superoxide dismutase is an enzyme found in aerobic cells which converts superoxide into hydrogen peroxide and molecular oxygen. SOD is found in two forms, one in the extramitochondrial cytosol and another in the mitochondria. The mitochondrial superoxide dismutase of eukaryotes is similar to the SOD of many bacteria with respect to its characteristic content of M_n^{2+} and many homologies in amino acid sequence. The cytosol form of SOD has quite a different structure and contains Cu^{2+} and Zn^{2+}. These enzymes are present in high concentration and are extraordinarily active.

Animal Toxicity of Paraquat

The toxicity of paraquat has been studied in many animal species including rats (Kimbrough and Gaines, 1970; Robertson et al., 1971; Short et al., 1972), rabbits (Butler and Kleinerman, 1971), guinea pigs and monkeys (Murray and Gibson, 1972). Rats and monkeys exhibit lung damage similar to that observed in humans, while rabbits appeared to be resistant to paraquat and failed to develop the lung lesion described. The absorption of ^{14}C-paraquat in rat after oral administration was poor, with elimination occurring in the urine and faeces (Daniel and Gage, 1966). No radioactivity appeared in the bile. Lichtfield et al., (1973) observed that an intravenous

injection of paraquat was rapidly distributed in most tissues except the brain and spinal cord. After 24 hours, however, paraquat was selectively concentrated in the lung and skeletal muscle from where it was slowly excreted.

The pathology of the lung lesion in rat has been extensively studied. The primary change found in the rat lung consists of vacuolization and degeneration of the membranous pneumocytes (type I), followed by increase of collagen and reticulin in the basement membrane and proliferation of granular pneumocytes (type II). Swelling of the endothelial cells, proliferation of fibroblasts, and increased numbers of endothelial cells follow. A well developed lung lesion shows areas where alveoli are completely obliterated or filled with an amorphous material which either formed a lattice or a large whorl of a very electrodense material similar to the material observed in the lamellar bodies (Kimbrough and Linder, 1973).

Paraquat has also been found to cause centrilobular necrosis in the rat liver and proximal tubular necrosis in the kidney (Murray and Gibson, 1972). Atrophy of the thymus has been noted in rats and rabbits (Butler and Kleinerman, 1971), and rats.

Mechanism of Paraquat Action

The mechanism of paraquat's toxic effects on the main target organ, lung is not known. In 1967, Manktelow put forward the hypothesis that paraquat specifically interfered with the production of pulmonary surfactant. This conclusion was essentially based on histopathologic findings in paraquat poisoned animals. The tissue alterations resembled pathologic features observed in some cases of

human respiratory distress syndrome. The absence of stable bubble formation over thick tissue sections corroborated that surface active material was decreased. This view has been shared by Robertson et al., (1970) and Fisher et al., (1969 and 1972). On the other hand, Fletcher and Wyatt (1970 and 1972), found that the phospholipid composition of rat lungs was unchanged after treatment with paraquat and that paraquat neither affected the amount of dipalmitoyl lecithin, the major lecithin constituent present, nor its rate of destruction. Thus, the effect of paraquat on surfactant material in the lung is unclear.

Lipid Peroxidation has been defined as the oxidative deterioration of polyunsaturated lipids. Paraquat and other related bipyridylium compounds have been investigated in the past and shown to be capable of being reduced in solution to give intensely colored, relatively stable free radicals (Michaelis and Hill, 1933). This property was shown to be a preprequisite for herbicidal activity (Calderbank, 1964). In the presence of oxygen, these radicals are rapidly reoxidized to the parent cations with production of superoxide radical ions (0_2^-), and hydrogen peroxide (Calderbank, 1964; Farrington et al., 1973). Paraquat is reduced by photosystems present in green leaves of plants, and cyclic redox reactions then lead to production of reactive oxygen species which are thought to be the molecules responsible for herbicidal activities.

Bus et al. (1974), present in vitro and in vivo evidence that mammalian toxicity of paraquat may be the consequence of cellular lipid peroxidation. The peroxidation being mediated through the single electron reduction of paraquat catalysed by NADPH-Cytochrome c

reductase with the subsequent transfer of the electron from reduced paraquat to molecular oxygen to form superoxide anion. The superoxide may non-enzymatically dismutate to form singlet oxygen which reacts with unsaturated fatty acids to form fatty acid hydroperoxides. Lipid free radicals which form spontaneously from lipid hydroperoxides, react with membrane polyunsaturated lipids with the formation of more lipid free radicals, thus continuing the chain reaction process of lipid peroxidation. The consequence of such free radical catalysed peroxidation is extensive damage to cell membranes with resultant loss of functional integrity. Paraquat and other bipyridylium compounds, like Diquat, can be reduced to free radicals by homogenates of liver, kidney, or lung (Gage, 1968; Baldwin et al., 1975). Incubation of paraquat with liver microsomes or a system containing NADPH-cytochrome c reductase, NADPH, and microsomal lipid greatly increase the formation of malonaldehyde which is dependent upon the concentrations of paraquat in the incubation mixture. However, Illet et al. (1974), found that paraquat inhibited in vitro lipid peroxidation.

It has been demonstrated that paraquat toxicity is significantly enhanced in selenium or vitamin E deficient mice or mice pretreated with dimethylmaleate and there is partial protection against paraquat toxicity by pretreatment with the enzyme, superoxide dismutase (Bus et al., 1974).

During the last few years, considerable attention has been focused on the pathological and biochemical changes brought about in lung tissue by oxygen and the oxidant gases, nitrogen dioxide and ozone. There are several recent reviews on the mechanism of the anatomic-pathologic and biochemical consequences of oxygen toxicity in

in the lung (Pfister and Nogues, 1974; Clark and Lambertsen, 1971; Haugaard, 1968). It is generally thought that lipid peroxidation may be an important consequence of exposure to normobaric or hyperbaric oxygen. It is interesting to note that the pathological findings in the paraquat lung have been likened to changes in the lung following oxygen toxicity. It would seem that, at normal oxygen tension, only small amounts of superoxide are formed and the endogenous enzyme, superoxide dismutase (SOD) is sufficient to degrade it to peroxide. But, when breathing pure oxygen, the amount of superoxide formed exceeds the capacity of endogenous superoxide dismutase to inactivate it, and then the superoxide is free to damage cellular components leading to cell death. The corrollary to paraquat poisoning is that the lung is exposed to higher concentrations of paraquat than any other tissue, and also achieves higher levels of paraquat. Thus, it is possible that even at normal oxygen tensions this may lead to the formation of more superoxide in the lung than the available superoxide dismutase can metabolise.

Non-Respiratory Functions of the Lung

The long held traditional view that the lung is solely and passively involved in gaseous exchange has been profoundly altered by contemporary research which has established its capacity to perform various metabolic functions.

The location of the lung in the body is strategic for modification of drug action. The lungs are the first visceral organs to receive parenterally administered drugs, thus, the uptake or metabolism of a drug by the lung may greatly affect the action of the drug.

The ability of the lung to modify the biological activity of substances passing through the pulmonary circulation has been referred to as the pharmacokinetic function of the lung. Thus, the lung is capable of altering the biological activity of many substances, endogenous or exogenous, brought to it via the blood. The lung has been shown to affect many vasoactive substances by degrading them or converting an inactive product to its active form. Thus, angiotensin I is converted into its active form, angiotensin II. The degree of inactivation seems to be specific for a given substance, ranging from almost complete inactivation of bradykinin (Farreira and Vane, 1967), 5-hydroxytryptamine (Thomas and Vane, 1967), prostaglandins E_1, E_2, and $F_2\alpha$ (Ferreira and Vane, 1967), and minor inactivation of norepinephrine (Ginn and Vane, 1968), to relatively free passage of compounds like epinephrine and angiotensin II (Hodge et al., 1967). These phenomena are often quantitatively sufficient to markedly modify the concentration of some circulatory substances and to create large arterio-venous differences.

These pharmacokinetic functions of the lung seem to chiefly reflect the metabolic activities of the endothelial cells of the pulmonary vasculature, and have been known to be affected by age, pregnancy, gaseous anesthetics and heart-lung by-pass (Junod, 1975).

The lung is also capable of acting as an endocrine organ, releasing into the circulation a variety of active substances including histamine, SRS-A and prostaglandins in response to stimuli such as anaphylaxis, peptides, and physical deformation. The significance of such release has not been fully investigated, but it is clear

that the lung provides an essential control of the blood levels of many biologically active substances.

The Handling of Paraquat and Other Amines by the Lung

Several classes of drugs seem to be concentrated preferentially in lung tissue. Localized high concentrations may result in selective or even generalized toxicity. It has been suggested that in some cases, the lung acts as a depot which buffers the remainder of the body from high concentrations of a compound. An example are the phenothiazines, which almost always appear in pulmonary tissue in high concentrations. The depot in the lung could maintain blood levels for several days.

In 1968, Vane reviewed the role of the lung in clearing circulating endogenous and exogenous substances. He listed basic, lipophilic amines as the compounds most likely to be concentrated in the lung. 5-hydroxytryptamine and imipramine are examples of amines whose handling by the lung have been extensively studied. The uptake of these compounds occurs by different saturable mechanisms and the Km and Vmax values are thirty and one hundred times greater for imipramine suggesting basic differences in terms of the affinity and the number of the sites for uptake (Junod, 1975). The administration of another basic amine with similar physicochemical properties can result in release of previously bound imipramine and this may result in unwanted side effects. The lungs appear to have various means of processing and of eliminating drugs. As already discussed, amines are taken up by the lung via what appears to be an active transport mechanism of endothelial cells. Similarly, prostaglandins E and F

series are taken up and degraded but the site of the metabolic reactions are not yet known. The lungs also process steroid hormones (Hartiala, 1974), apparently for their own use, but it may be significant that the lungs can convert cortisone to its more potent analog, cortisol. Some of the cortisol thus formed leaves the lung and enters the arterial circulation.

The Handling of Propranolol and Other β-Adrenergic Agents by the Lung

Two main types of β-adrenergic receptors have been identified. These are β-1 receptors in the heart and β-2 receptors in the trachea, bronchi, and blood vessels. Isoproterenol seems to stimulate both β-1 and β-2 receptors. Salbutamol, carbuterol, and fenoterol stimulate β-2 receptors (Cullum et al., 1969; Giles et al., 1973; Wardell et al., 1974). The β-blocking agents include propranolol, bunolol, sotalol, and KÖ592.

Propranolol is the most commonly used β-adrenergic blocking agent. The aliphatic hydroxyl group on the propranolol molecule appears to be essential for activity and it gives the molecule its optical activity. The l-form is more potent than the d-form, and this difference is used to distinguish β-blockade from those other pharmacological actions of the molecule, such as, local anesthetic effect. Propranolol is well absorbed after oral administration. It is concentrated in the lung, and to a lesser extent, in the brain, liver, kidney and heart and excreted in urine after being almost completely metabolized. Two main metabolities, naphthoxy lactic acid and 4-hydroxypropranolol are found. 4-hydroxypropranolol is found only after oral or intraperitoneal administration and has blocking

activity similar to that of propranolol but with a shorter duration of action.

Junod (1975) reported on studies of dl-propranolol accumulation in the rat lung. It is a saturable process and compounds with similar physicochemical properties can inhibit the binding or accelerate the release of bound propranolol. There is, however, partial Na^+ dependence and marked temperature dependence at low substrate concentrations.

The β-Adrenergic System and Cyclic Nucleotides

The key compound that is involved in the mediation of most of the metabolic effects of the β-adrenergic system (as well as a great number of hormones) is cyclic 3',5', adenosine monophosphate (c-AMP). This compound was first described in 1957 by Sutherland as a co-factor for the conversion of liver phosphorylase from its inactive to its active form, allowing liver glycolysis to proceed (Cleveland et al., 1972). c-AMP is formed from adenosine triphosphate (ATP) by the action of adenyl cyclase, an enzyme located within the cell membrane of the target cell, and it is inactivated (hydrolysed to 5' AMP) by phosphodiesterase (PDE), a soluble cytoplasmic enzyme.

$$\text{ATP} \xrightarrow{\text{AC}} \text{c-AMP} \xrightarrow{\text{PDE}} \text{5' AMP}$$

The equilibrium concentration of c-AMP, therefore, depends on the relative activities of these two enzymes. The activity of adenyl cyclase is increased by a number of factors including adrenergic stimulation while PDE is inhibited by xanthine derivatives,

particularly theophylline, so that either may result in an increase in c-AMP levels.

Cyclic AMP acts not only as a second messenger for the effects of nearly all hormones, but also, together with the other known natural cyclic nucleotide, cyclic guanosine 3',5'monophosphate (c-GMP), appears to be intimately involved in the control of almost all facets of cellular activity (Robison, et al., 1971; Greengard et al., 1972; Hardman et al., 1971).

PURPOSE

The overall purpose of this study was to determine the effect of other chemical agents on the toxicity of paraquat and how these could be adapted for use in the management of paraquat poisoning.

The objectives were three fold. The first was to study the effect of paraquat on the isolated perfused rat lung preparation, and how its effects may be modified by other chemical agents; and second, to conduct acute and chronic *in vivo* experiments to determine the effect of other agents on paraquat toxicity. The third objective was to study how the paraquat affected lung handles endogenous substances such as the prostaglandins, peptides and biogenic amines which are effectively regulated by the normal lung.

METHODS

Animals

Adult male Sprague-Dawley rats (from Spartan Research Animals, Inc., Haslett, Michigan), weighing 150 - 250g were used in all experiments. The animals were housed in plastic cages in groups of three and were allowed food and water ad lib. The room temperature was maintained at 21 - 24°C and the light - dark cycle was twenty-four hours.

Perfusion

The perfusion apparatus is shown in Figures 1 and 2. In this set up for the isolated perfused lung experiment, the conditions under which the lung is normally maintained in the chest of the animal were simulated. This involved placing the lungs in an "artificial thorax" and connecting the trachea in such a way that gases can pass in and out of the lungs. The "artificial thorax" is a sealed chamber maintained at normal body temperature (37°C). The chamber was arranged in such a manner that the isolated lung can be suspended in it and can be subjected to alterations that are somewhat similar to the changes in pressure that the lungs undergo while in the thorax of the intact animal. The changes in pressure in the "artificial thorax" were produced by connecting the sealed chamber to a pump (Rodent respirator pump by Harvard Apparatus Co.), that will alternate the pressure

Figure 1. Lung Perfusion Apparatus--showing general outlay.

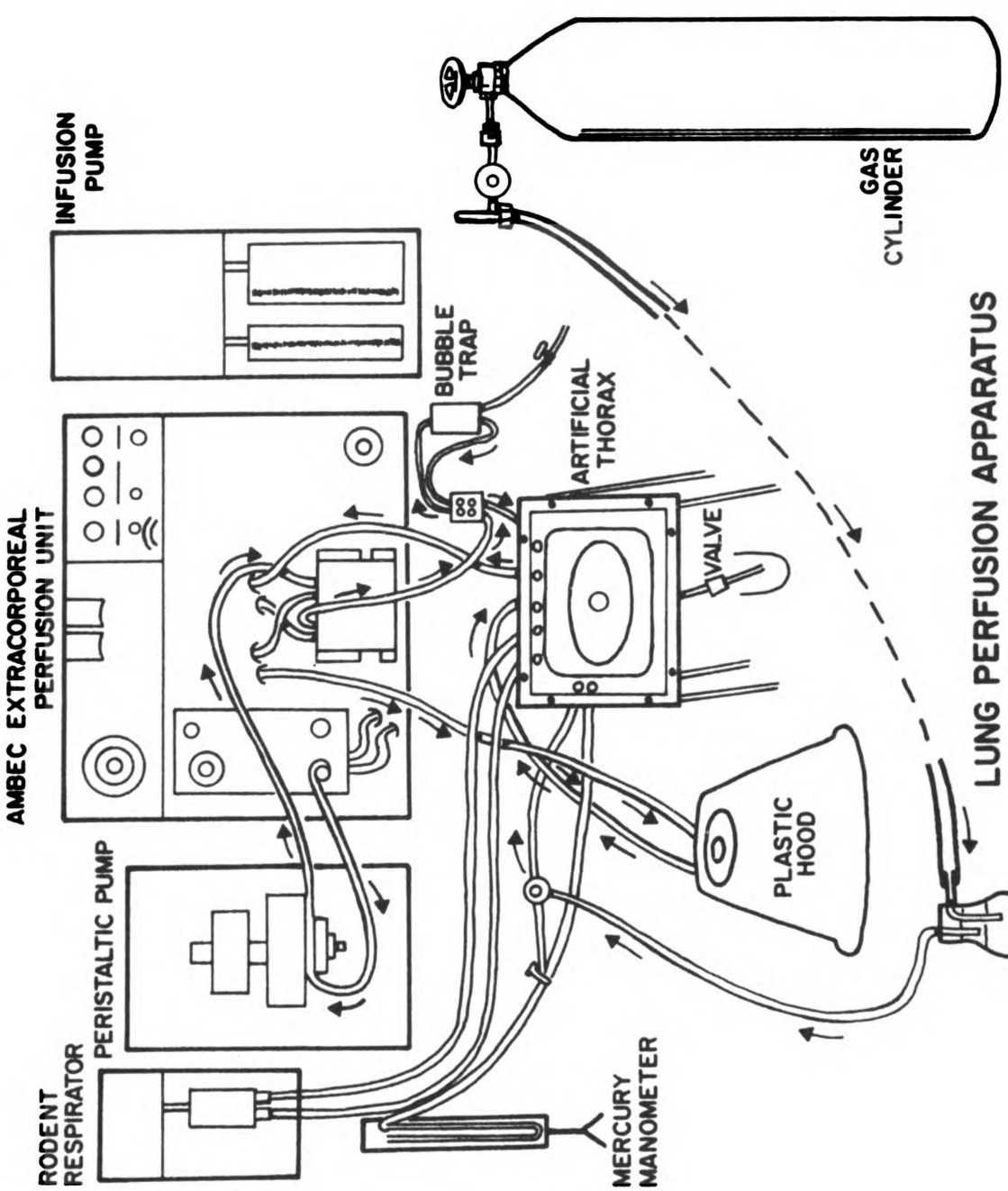
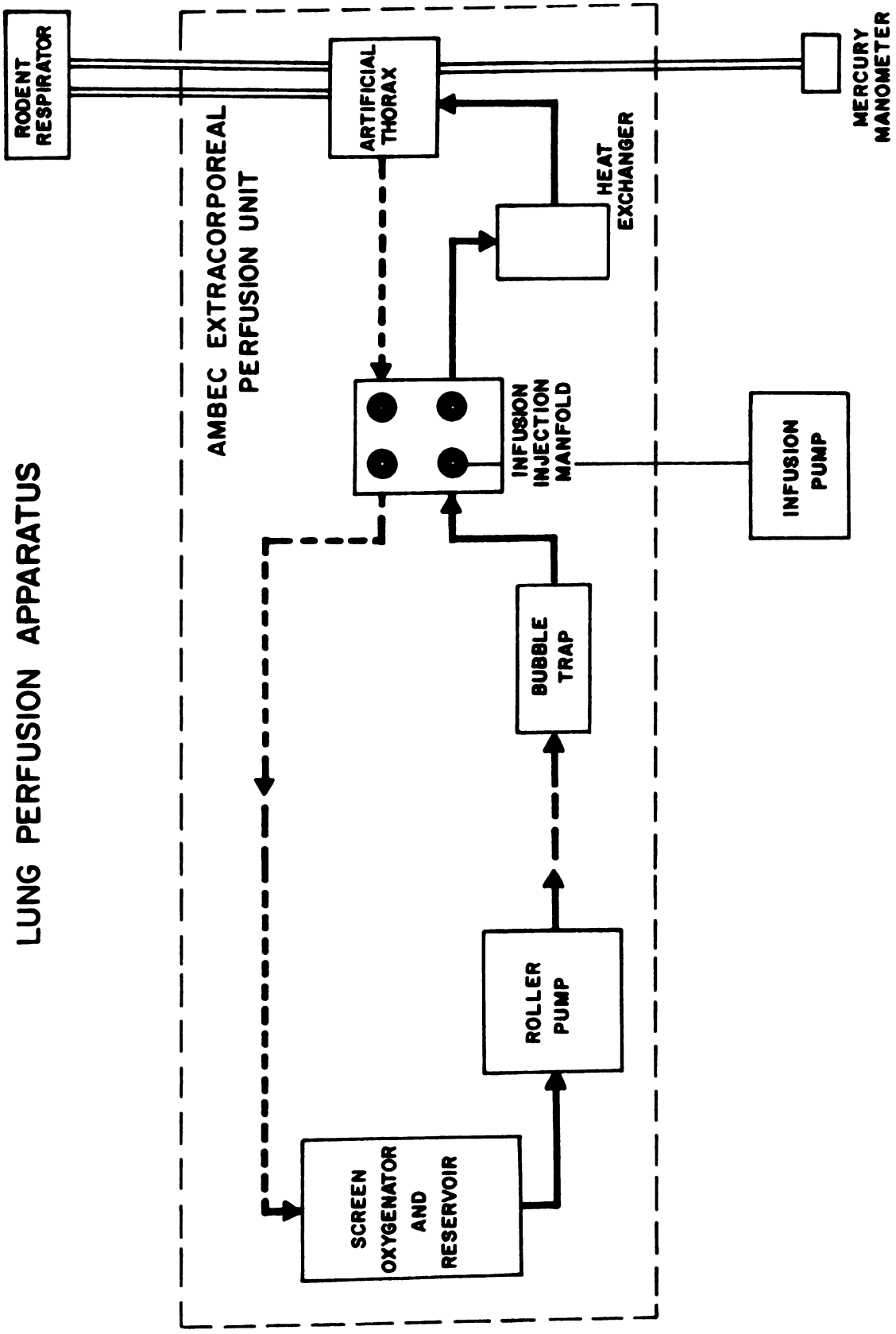

INFUSION PUMP
AMBEC EXTRACORPOREAL PERFUSION UNIT
BUBBLE TRAP
ARTIFICIAL THORAX
VALVE
GAS CYLINDER
PERISTALTIC PUMP
RODENT RESPIRATOR
MERCURY MANOMETER
PLASTIC HOOD
LUNG PERFUSION APPARATUS

Figure 2. Lung Perfusion Apparatus--showing diagram of perfusate flow during perfusion.


LUNG PERFUSION APPARATUS
AMBEC EXTRACORPOREAL PERFUSION UNIT
RODENT RESPIRATOR
ARTIFICIAL THORAX
MERCURY MANOMETER
HEAT EXCHANGER
INFUSION INJECTION MANFOLD
INFUSION PUMP
BUBBLE TRAP
ROLLER PUMP
SCREEN OXYGENATOR AND RESERVOIR

within the sealed chamber between 2mm Hg and -9 to -10mm Hg. This necessitated that the chamber be airtight and that a one-way valve be inserted into the perfusate outflow tract. The plastic chamber contained three outlets: one was used to connect tubing from the respirator pump, another was used to connect tubing from the manometer, and the third was extra. The pressure in the chamber was monitored via the mercury manometer. The lungs responded by inspiratory and expiratory movements.

The Ambec Extracorporeal Perfusion Unit (Beck Industries Inc., Boulder, Colo. 80302), consists of a screen oxygenator and reservoir for the perfusate, a bubble trap and an infusion injection manifold which allows the injection of drugs and chemicals under specific conditions. The whole perfusion apparatus was thermostatically kept at 37°C. The perfusion flow rate through the lungs was determined by measuring the time required for the perfusate to fill a 10ml calibrated tube located below the funnel-shaped floor of the chamber. The perfusion flow rate was kept at 10ml per minute and the perfusion pressure was 12-15mm Hg.

Surgery

The rat was weighed and anesthetised with pentobarbital (50mg/kg), given intraperitoneally. The trachea was exposed, cannulated with a T-shaped stainless steel tube (ID .070") and secured in place. The abdomen was then opened and the inferior vena cava was exposed and heparin (0.6ml of 1,000 units/ml) was injected into it. The heparin was injected to prevent the formation of clots in the pulmonary vessels.

After allowing two minutes for the heparin to act, the rat was bled by excising the abdominal aorta in the lower part of the abdomen. This facilitated the cannulation of the pulmonary artery by diverting blood from the thorax into the abdomen.

The trunk of the pulmonary artery was cannulated by making an incision in the right ventricle close to its origin and a plastic cannula (ID .062") was inserted and secured in position. The lungs were flushed with Kreb's Ringer bicarbonate solution and then carefully dissected from the heart and other adjacent organs in the thoracic cavity and suspended in the "artificial thorax" by means of the trecheal cannula. The chamber was then sealed and the pressure inside was alternated between -2mm Hg and -10mm Hg with the roden respirator. The whole procedure was carried out usually within 6 to 7 minutes.

Perfusate

The lung was perfused with Kreb's Ringer bicarbonate solution containing bovine serum albumen (3%) and dextrose (0.15%) via the pulmonary artery and the perfusate was allowed to flow freely from the veins.

The lungs were ventilated with gas mixture made up of 95% of oxygen and 5% of carbon dioxide. The gases (95% O_2; 5% CO_2) inspired by the lungs were warmed and moistened by bubbling through distilled water maintained at 37°C. The warm moistened gases enter the lungs via tubing that connects the humidifying chamber and the trachea. The flow of gases into and out of the lungs was regulated by a series of check valves in the tubing. The check values were arranged so that the lung did not repeatedly inhale and exhale the same volume

of gases in the tubing, but rather inhale a new supply of gases each time the lungs complete the respiratory cycle.

Assays

Lung protein concentrations were determined by the Lowry total protein method (Lowry et al., 1951), reading the samples at 750 nm (or 660nm) on the Gilford spectrophotometer 240, (Gilford Instrument Laboratories Inc., Oberlin, Ohio, U.S.A.).

Paraquat Concentration in rat lung and perfusate was determined by a colorimetric method which is a modification of the method described by Ilett et al. (1974). 2.0 ml portions of 5M ammonium chloride eluate from Dowex-50 columns were assayed colorimetrically for paraquat by adding 0.5 ml of sodium dithionite (sodium hydrosulphite, 0.2% in 1N sodium hydroxide), mixed and the absorbance immediately read at 395 nm in a Gilford spectrophotometer 240. Paraquat dichloride (methyl viologen, Sigma Chemical Co., St. Louis, Missouri) was used to prepare the standard solutions.

Propranolol Concentration in the rat lung and perfusate samples were determined by a modification of the spectrofluorophotometric method of Black et al. (1965). The lung was homogenized in the Sorvall Omni-mixer (Ivan Sorvall Inc., Newtown, Connecticut 05470, U.S.A.), in 10% trichloroacetic acid (TCA) and then centrifuged. The resulting precipitate was then dissolved in 25 ml o 1N NaOH. 2ml of the solution were extracted with 6 ml of heptane containing 1.5% isoamyl alcohol, centrifuged, and the lower (aqueous) layer discarded. 1.5 ml of 0.1N HCL was added to the upper (organic) phase and centrifuged again. The resulting lower phase (acidic extract) was read in

the Aminco-Bowman Spectrophotofluorometer (American Instrument Co., Inc, Silver Springs, Maryland) at 295 mμ (maximum excitation) and 360 mμ (minimum emission).

Theophylline Concentration in the lung and the perfusate was determined by a modified spectrophotometric method described by Schack and Waxler (1949). 4 ml portions of the supernatant from the lung homogenate were extracted with 0.4 M phosphate buffer solution (pH 7.4), NaCl, and ethyl-ether-$CHCL_3$-isopropyl alcohol (100:100:10:1), centrifuged, and 10 ml of the separated upper phase was mixed with 0.05 M glycine buffer solution (pH 10.6) and centrifuged again. Volatile solvents were removed from the separated upper layer (aqueous) phase and its extinction measured at 275 nm in the Gilford spectrophotometer 240. A blank was treated similarly in each case.

Cyclic AMP (c-AMP) in the lung and perfusate was determined by a competitive binding protein assay method based on the method described by Brown et al. (1971). The lung was homogenized in the Sorvall Omni-mixer in 25 ml of 10% trichloroacetic acid and then centrifuged. 10 μl of radioactive c-AMP (^{3}H-c-AMP) was added to the supernatant and the resulting mixture was extracted five times with an equal volume of ether saturated with water. The resulting sample was then frozen in dry ice and acetone, and lyophilized. The precipitate was redissolved in Tris/EDTA and assayed for c-AMP.

Cyclic GMP (c-GMP) in the lung and perfusate samples was determined by using iodinated c-GMP derivative as tracer in the method described by Steiner (1972). A portion of the supernatant after homogenizing the lung in 25 ml of 10% trichloroacetic acid was treated with ^{3}H-c-GMP and extracted five times with equal volumes of

ether-saturated water. One-ninth sample volume of 50 mM Tris-HCl buffer (pH 7.0) was then added and the resulting sample applied to alumina column previously washed with 10 ml of 5 mM Tris buffer. The eluate was applied to an Ag 1x8 column, washed with 4N formic acid and the final eluate frozen in acetone and dry ice, and then lyophilized. The lyophilized specimen was reconstituted in sodium acetate buffer and assayed.

Glucose Studies

The disappearance of glucose from the perfusate was followed in 6 perfusion experiments. Six 1 ml samples were taken at 15 minute intervals over a 90 minute period. Glucose in these samples was determined by the Glucostat Method. The experiment was conducted with the lung in position in one group, and without lung in the controls.

Paraquat Studies

Bovine serum albumin--Kreb's Ringer bicarbonate solution containing different concentrations of paraquat was perfused through the rat lung for 90 minutes. The lungs were weighed before and after the experiments to obtain the increase in weight. The drugs, mannitol, propranolol, and histamine were also infused side by side with paraquat in separate experiments to determine their effects on the paraquat toxicity as measured by percentage weight increase of the lung over the 90 minute period.

Separate control experiments were conducted with histamine, propranolol, and BSA-Kreb's Ringer solution to find their effects on the lung in the absence of paraquat.

Effect of Paraquat on Rat Lung Cyclic AMP and Cyclic GMP

The rat lung was isolated and mounted as before and perfused with bovine serum albumin for 10 minutes for stabilization. The lung was then infused with isoproterenol, propranolol, or paraquat for 90 minutes. Perfusate specimens were taken after 5 minutes, 10 minutes, 15 minutes, and 20 minutes of infusion. At the end of the experiment, the lung was frozen in liquid nitrogen and later homogenized in 10% trichloroacetic acid and centrifuged at 5,000 rpm. Portions of the supernatant were used for cAMP, cGMP, and protein determinations.

Rat Isolated Lung Perfusion Experiment

To investigate the lung handling of endogenous substances, prostaglandins E_2, $F_2\alpha$ and A_1.

Control Studies: The isolated lung perfusion apparatus was set up as described before but no lung was included. The system was then perfused with BSA-Kreb's Ringer solution for 10 min for stabilization. In separate experiments, BSA-Kreb's Ringer solution containing radioactive PGE_2 (^{3}H-PGE_2) was infused using non-circulating arrangement and collecting perfusate specimen in 1 min portions into 1 ml of 10% trichloroacetic acid (TCA).

The above experiment was repeated using 4 lungs from normal rats in each set of experiments for a particular prostaglandin (lungs A to D). Lungs A and B were infused for 5 min and lungs C and D for 10 min.

Paraquat-Damaged Lung Studies: The above experiments were repeated using lungs from rats which had been treated with paraquat (5 mg/kg daily) for 8 days. At the end of each experiment, the lung

was blotted dry with gauze, weighed and homogenized in acteone-methanol (1:1) and 0.2 ml of the homogenate was introduced into a counting bottle and evaporated to dryness on a hot plate in the fume chamber. The residue was then redissolved in 0.2 ml of distilled water and 10 ml of ACS (aqueous counting scintillant) was added and radioactivity counted. The remaining lung homogenate was then centrifuged at 5000 rpm for 20 min and nitrogen was blown over the solution to dryness. The resulting precipitate was dissolved in alcohol (200% proof) and drops applied to thin plate chromatogram. The perfusate was centrifuged after the addition of 2 ml of 10% TCA. 0.2 ml of the supernatant was removed and 1 ml of tissue solubilizer soluene-100 Packard Instrument Co. Inc., Ill. 60515) added and the vials left in the oven for about 2 hrs. Then, 10 ml of ACS was added and radioactivity determined in Mark II Scintillation counter (Searle & Co., Des Plaines, Ill. 60018).

Rat Isolated Lung Perfusion Experiment: To investigate the lung handling of endogenous substances, prostaglandins (PGE_2).

Ten rats were divided into two groups. One group was treated with 0.9% saline ip for eight days. The second group received paraquat (5mg/kg) ip for eight days. On the eighth day of the experiment, the rats were anesthetized with sodium pentobarbital and their lungs removed and mounted in the "artificial thorax" as described previously.

After allowing 10 min equilibration, 0.1ml perfusate containing 0.25 µCi of ^3H-PGE_2 (specific activity = 117 Ci/M.mole, New England Nuclear), was injected via the infusion injection manifold and the venous effluent collected for 1 min (one circulation time). The experiment was carried out with 5 lungs from saline and 5 lungs from paraquat treated rats.

Separation and Assay of PGE_2 and Metabolites: PGE_2 and its metabolites in both venous effluent and lung homogenate were separated by both thin layer (TLC) and silicic acid chromatography and assayed.

Separation by TLC: After perfusion each lung was homogenized in 3ml of Na-phosphate buffer (0.1M, pH 7.4, containing 0.15M NaCi). To 3ml of perfusate or lung homogenate in 40ml siliconized graduated glass centrifuge tubes was added 9ml of a mixture of ethyl acetate, isopropanol and 0.1M HCl (3:3:1 by vol), 6ml of ethyl acetate and 9ml of water. After mixing and centrifuging at 2000 rpm for 10 min, the upper organic phase (3 to 3.5ml) was transfered into siliconized counting vial and dried at 55°C in a stream of nitrogen gas (Jaffe and Behrman, 1974). The precipitate was dissolved in 100 µl of absolute alcohol and 20 µl portions were applied to silica gel G thin-layer plates (Analtech, Newark, Del.).

Twenty µl portions of non-radioactive authentic standards of PGE_2, 15-keto-PGE_2, 13,14-dihydro-15-keto-PGE_2 and 13,14-dihydro-PGE_2 (Upjohn Co., Kalamazoo, MI.), were applied separately on one side of the plate and the samples from lung homogenates and lung venous effluent on the other side. The plates were developed in chloroform: tetrahydrofuran acetic acid (100:10:5 by vol) for 80 min. The solvent front traveled 15cm during this time. The plates were allowed to dry for 15 min at room temperature and the chromatograms of the prostaglandin standards were sprayed with 5% phosmolybdic acid and dried at 120°C for 15 min. The silica gel was scraped in sections (1x1 cm) into scintillating vials containing aqueous counting scintillant (ACS-Amersham/Searle) and the radioactivity present was determined in

a Mark II Liquid Scintillation Counter (Searle & Co., Des Plaines, Ill. 60018).

Separation and Assay of PGE_2 and Metabolites by Silicic Acid Chromatography: A slurry of silicic acid, 100 mesh (Malinckrodt Chemical Works, St. Louis, Missouri) was prepared in benzene:ethyl acetate (60:40 v/v, 0.25 gm silicic acid/ml). Two ml of slurry was poured into 10ml disposable pipettes with glass wool inserted at their tips. The column was then washed with 5ml of benzene:ethyl acetate:methanol (60:40:20) and 1.5ml benzene:ethyl acetate (60:40).

Sample of venous effluent or lung homogenate dissolved in 0.2ml benzene:ethyl acetate:methanol (60:40:10) and 0.8 ml of benzene: ethyl acetate (60:40) was placed on the silicic acid column and fraction 1 was collected. Three other fractions were collected after washing the columns with 10ml of benzene:ethyl acetate (60:40) (Fraction 2), 18ml of benzene:ethyl acetate:methanol (60:40:20) (Fraction 3) and 6ml of benzene:ethyl acetate:methanol (60:40:20) (Fraction 4). All specimen were dried under nitrogen stream at 55°C and 10ml of ACS added to each vial and radioactivity present was determined in Mark II Liquid Scintillation Counter.

Rat Chronic In Vivo Experiments

In these experiments, rats were treated with different chemical agents including paraquat, propranolol, theophylline, and saline in different combinations. After a specified number of days, a number of rats from different groups were killed, their lungs removed as described for the isolated lung experiments. The liver, spleen, kidney, thymus, and heart were also taken from the rats in some cases.

These specimens were assayed for total proteins, cyclic AMP, cyclic GMP and the appropriate drug or drugs the rat had been treated with. Specimens for cyclic AMP and cyclic GMP were kept frozen in liquid nitrogen.

Rat Chronic In Vivo Experiment I

Two groups of thirty male rats were used. One group was treated once daily with normal saline (0.05 ml/10g body weight) intraperitoneally, and the second group was treated once daily with 5 mg/kg ip of paraquat. The rats were weighed daily before their treatments. Five rats from each group were killed on the 3rd, 6th, 8th, 10th, and 12th days of the experiment. The lungs were removed, homogenized and assayed for cyclic AMP, cyclic GMP, paraquat, and total proteins.

Rat Chronic In Vivo Experiment II

Six groups of rats were used in this experiment. The groups were made up as below (groups receiving paraquat had five more rats).

Group	Treatment	No. of Rats	Dose of Drug Used
A	Saline (0.9% NaCl) Treated Rats	25	0.5 ml/10G weight ip
B	Paraquat Treated	30	pq-5mg/kg ip; prop-5-mg/kg/s.c.
C	Paraquat and Propranolol	30	pq-5 mg/kg ip; prop-5 mg/kg s.c.
D	Propranolol Treated	25	prop 5 mg/kg s.c.
E	Theophylline Treated	25	Th 10 mg/kg ip
F	Paraquat and Theophylline Treated	30	pg-5 mg/kg; Th-10 mg/kg ip

The injections were given daily and the rats were weighed each time before the injections. Five rats from each group were killed on the 3rd, 6th, 8th, 10th, and 13th days of the experiment. The lungs were removed, homogenized and assayed for total proteins, paraquat, cyclic AMP, cyclic GMP, theophylline and propranolol concentrations.

Rat Chronic In Vivo Experiment III

Five groups of ten rats were used in this experiment. All the rats were killed on the 9th day, unlike Experiment II in which groups of 5 rats were killed on 3rd, 6th, 8th, 10th, and 13th days. The group of rats were treated as below:

Group	Treatment	No. of Rats	Dose of Drug Used
B	Paraquat	10	5 mg/kg body weight ip
C	Paraquat and Propranolol	10	pq-5 mg/kg; prop-5 mg/kg sc
D	Propranolol	10	5 mg/kg tid sc
E	Theophylline	10	10 mg/kg ip
F	Paraquat & Theophylline	10	pq-5 mg/kg; Th-10 mg/kp ip

The rats were injected daily after being weighed. Paraquat treatment was given daily in the morning and propranolol was given three times a day at equally spaced intervals. On the 9th day, all rats were anesthetized and their lungs removed and kept frozen in liquid nitrogen. These lung specimens were homogenized and assayed for cyclic AMP, cyclic GMP, paraquat, theophylline and propranolol concentrations.

Rat Chronic In Vivo Experiment IV

Experiments to compare the effect of l-propranolol and d-propranolol on paraquat toxicity.

The groups were treated as below:

Group	Treatment		Dose of Drug Used
B	Pq	Rats 1 to 5	Pq 15 mg/kg stat. ip
		6 to 10	Pq 10 mg/kg stat. ip
C	Pq+l-Prop.	Rats 1 to 5	Pq 15 mg/kg stat. + l-Prop 5 mg/kg tid sc
		6 to 10	Pq 10 mg/kg stat. + l-Prop 5 mg/kg tid sc
D	Pq + d-Prop.	Rats 1 to 5	Pq 15 mg/kg stat + d-Prop 5 mg/kg tid sc
		6 to 10	Pq 10 mg/kg stat. + d-Prop 5 mg/kg tid sc
G	l-Prop.	Rats 1 to 10	l-Prop. 5 mg/kg tid sc
H	d-Prop.	Rats 1 to 10	d-Prop. 5 mg/kg tid sc

The rats were weighed daily before injection. On the 9th day, all the rats were killed and their lungs removed as described before. The lungs were homogenized in 25ml of 10% trichloroacetic acid and divided into 5 portions for cyclic AMP, cyclic GMP, total proteins and paraquat determinations.

The lung precipitate was dissolved in 25ml of 1N NaOH and 2ml samples were used for propranolol determination.

Rat Acute In Vivo Experiment I

Two groups of rats were used in this experiment. The control group was made up of 45 rats and the paraquat treated group of 40 rats. Rats in the control group were treated with saline (0.9% NaCl)

ip and rats in the paraquat treated group receivd 20 mg paraquat cation (27.6 mg of paraquat dichloride) per kg of body weight. Five rats from each group were killed at intervals as shown below.

Time	Control Group	Paraquat Treated Group
1. 0.00 Hr	5 Rats--No injection	Nil
2. 0.50 Hr	5 Rats--0.5ml/kg saline injected	5 Rats injected with Pq 20 mg cation ip
3. 1.00 Hr	5 Rats-- " " "	5 Rats " " "
4. 1.50 Hr	5 Rats-- " " "	5 Rats " " "
5. 2.00 Hr	5 Rats-- " " "	5 Rats " " "
6. 4.00 Hr	5 Rats-- " " "	5 Rats " " "
7. 8.00 Hr	5 Rats-- " " "	5 Rats " " "
8. 16.0 Hr	5 Rats-- " " "	5 Rats " " "
9. 24.0 Hr	5 Rats-- " " "	5 Rats " " "

At the appropriate time, each rat was weighed, anesthetized with pentobarbital, intubated and the lungs removed quickly and frozen in liquid nitrogen. The lungs were homogenized in the Sorvall Omni-mixer and assayed for the concentrations of total proteins, paraquat, cyclic AMP, and cyclic GMP.

Rat Acute In Vivo Experiment II

Experiment: To determine the effect of reserpine and atropine on paraquat induced increase in rat lung cyclic nucleotides.

Three groups of rats were used in this experiment. Each group was pretreated with saline (0.05 ml/10g) ip daily for two days, reserpine (50 mg/kg) ip daily for two days, or atropine sulfate

(0.5 mg/kg) ip for four hours. The pretreatments were followed by injection of paraquat cation 20 mg/kg ip. Then five rats from each group were killed at 30 minutes, 1 hour, and 2 hours after paraquat injection. The control rats received no paraquat. Each rat was anesthetized with pentobarbital, and the lungs removed as described before and quickly frozen in liquid nitrogen. The lungs were homogenized and used to assay for total proteins, cyclic AMP and cyclic GMP.

Analysis of Results

In the isolated lung perfusion and the acute *in vivo* experiments, values for lung total proteins were expressed in mg and cyclic AMP and cyclic GMP in picomoles (pmoles) per mg of protein. Concentrations of chemical agents used were expressed in appropriate units per mg of lung protein.

In the Rat Chronic *in vivo* experiments, values for lung total proteins were expressed in mg and cyclic AMP and cyclic GMP were expressed in pmoles per whole lung. The concentrations of drugs contained in the lung were expressed in the appropriate units per whole lung. Specific values cannot be expressed in terms of protein concentration because there is an increase in total lung proteins on chronic treatment of rats with paraquat.

Statistics

Statistical evaluation of the data was by the Student's t-test or analysis of variance (completely randomized design) with differences among means analysed by the least significant difference method

(Sokal and Rohlf, 1969). Binomial expansion (Goldstein, 1964) was used to test for significant difference in frequency of mortality between groups.

The level of significance was chosen as $p < 0.05$ in all cases.

RESULTS

Isolated Perfused Lung Experiments

General condition of the lung: Gross observation of the lung can provide a degree of measure of viability. In a dying lung, the organ becomes markedly edematous and grossly necrotic, and there is a sharp drop in the flow of perfusate.

The lungs in this study perfused with only bovine serum albumin-Kreb's Ringer bicarbonate solution appeared to remain viable by the above gross standards. The surface of the lung remained glistening and there were no gross signs of necrosis. Furthermore, flow rates remained constant throughout the perfusion period, and there were no signs of edema.

The effect of perfusion of a number of drugs on the weight of the rat lung was studied in the isolated perfused rat lung preparation. The lungs were weighed before and after perfusion. Values were expressed as the lung weight or the percent change in lung weight $\pm$ SEM. Values in parentheses refer to the number of lungs perfused (Table 1). Paraquat produced the biggest mean percent lung weight change (37.1 $\pm$ 17.4) during the 90 min perfusion period (Figure 4). In the presence of propranolol, the mean percent weight change was significantly reduced (1.4 $\pm$ 0.4). Perfusing paraquat in the presence of mannitol reduced the mean percent weight change to 6.5 $\pm$ 1.8.

Glucose Studies

Glucose present in the perfusate at the beginning of perfusion was about 0.15 g per 100 ml of bovine serum albumin--Kreb's Ringer bicarbonate solution. Glucose concentration in the perfusate declined as a linear function through the 90 minute perfusion period (Figure 5). To determine whether this disappearance was due to metabolism, experiments were conducted without including lungs and no change in the glucose concentration in the perfusate was observed over the period of 90 minutes (Figure 5). The slopes of the two lines were significantly different ($p < 0.05$).

Effect of Paraquat on Rat Lung Cyclic AMP

Perfusing the lung with bovine serum albumin--Kreb's bicarbonate solution containing paraquat (10^{-4} M) resulted in approximately a two fold increase in lung cyclic AMP. On perfusing paraquat in the presence of propranolol (10^{-4} M) there was a significant reduction in the cyclic AMP levels in the lung compared to levels achieved with paraquat alone. Perfusion with isoproterenol caused about a two and a half fold increase in lung cyclic AMP, and propranolol alone resulted in a significant reduction in the lung cyclic AMP compared to lung cyclic AMP values obtained on perfusing with only bovine serum albumin--Kreb's bicarbonate solution (Figure 6).

Rat Isolated Lung Perfusion Experiment

The Rf values for PGE_2 and metabolities are shown in Table 8. The compounds are easily separated with developing solvents adapted from Amersham (Searle System 60).

The total radioactivity recovered from the chromatogram plates after applying ^{3}H-PGE$_2$ were within 70-76% of the activity of the applied doses.

In experiments with no lung included in the system, the radioactivity recovered from the venous effluent after injecting ^{3}H-PGE$_2$ contained 87.7% PGE$_2$, 0.77% 15-keto-PGE$_2$ and 3.32% 13,14-dihydro-15-keto-PGE$_2$ and no radioactivity for 13,14-dihydro-PGE$_2$.

Table 9 shows the percentage of radioactivity recovered for ^{3}H-PGE$_2$, 15-keto-PGE$_2$ and 13,14-dihydron-15-keto-PGE$_2$ from the venous effluent and lung homogenates of both control and paraquat treated rats. No 13,14-dihydro-PGE$_2$ was detected.

Table 10 shows the extent of metabolism of PGE$_2$ and the related formation of the metabolites 15-keto-PGE$_2$ and 13,14-dihydro-15-keto-PGE$_2$ by TLC. It is clear from Table 10 that significantly less PGE$_2$ was metabolized and 15-keto-PGE$_2$ was formed by the lung from the paraquat treated rats compared to controls.

Data in Table 10 was derived from Table 9 by subtracting corresponding values from perfusion experiment without rat lung.

The results from silicic acid chromatrography were consistent with those from the analysis by TLC (Table 10).

Rat Chronic and Acute In Vivo Experiments

Rat Chronic In Vivo Experiment I: In this experiment, three rats died in the paraquat treated group and none in the control group. Of the three rats that died, two did so on the 11th day and one on the 12th (Table 2). The paraquat treated rats initially gained weight at

a slower rate compared to the controls but after the 8th day of the experiment, progressively lost weight (Figure 7). Total lung protein expressed in mg per lung showed a steady increase in rats treated with paraquat and by the 12th day, had achieved about two fold increase compared to rats which were treated with saline (Figure 8). The mean total protein concentrations in the paraquat treated group were significantly different from controls from the 8th day onwards (Table 3).

Lung cyclic AMP and cyclic GMP concentrations were significantly increased in the paraquat treated rats and the peak values of these increases occurred on the 8th day of the experiment and coincided with the highest level of paraquat concentration in the lung which also occurred on the 8th day. The cyclic AMP was increased two fold at the peak time and the cyclic GMP ten fold (Figures 9 and 10).

Rat Chronic In Vivo Experiment II: Six groups of rats were used in this experiment. The three control groups were made up of 25 rats, each received saline, propranolol, or theophylline. The other three groups (30 rats in each group) were treated with paraquat, paraquat and propranolol, or paraquat and theophylline. Four rats (13%) died in the paraquat treated group, one on the ninth day, two on the tenth day, and one on the eleventh day of the experiment. There were 6 deaths (20%) in the paraquat and propranolol group with one death on the ninth day, two on the tenth day, and three on the twelfth day. The highest number of deaths, 8 (27%), occurred in the paraquat and theophylline group. The frequency of death in the three groups which received paraquat was not significantly different ($p > 0.05$), but the deaths occurred earlier in the paraquat and theophylline

treated group than in the other groups referred to above (1 on the 7th day, 1 on the 8th day, 2 on the 9th day, 2 on the 10th day, and 3 on the 12th day) (Table 4). Body weight changes were also followed in the different groups (Figure 11). Rats in the groups receiving paraquat showed slower weight gain up to the 6th day and then a progressive decrease in weight with a significantly higher rate of weight loss in the group which received paraquat and theophylline compared to rats which received paraquat only or paraquat and propranolol. The three control groups showed a steady increase in weight.

The concentration of paraquat in the lungs of the different groups of rats which received paraquat was determined. The highest level of paraquat in the lungs was found on the 10th day in the groups which received paraquat. The group which received paraquat and theophylline had significantly ($p < 0.05$) higher level of paraquat in the lungs compared to rats which received paraquat only or paraquat and propranolol (Figure 12). Lung propranolol concentration was highest on the 3rd day in both the group which received only propranolol and that which received paraquat and propranolol (Figure 12). There was significantly higher concentration of propranolol in the lungs of rats which received paraquat and propranolol. Lung theophylline concentrations peaked on the 3rd day in rats which were treated with paraquat and theophylline, and on the 6th day in rats which received theophylline only. The theophylline concentrations at the two peaks in the two groups were not significantly different (Figure 13). Mean total lung cyclic AMP concentrations in all rats increased with time and the increases were highest in rats which received paraquat or paraquat and theophylline.

Rat Chronic In Vivo Experiment III: In this experiment, two rats from the group treated with paraquat and theophylline died (Table 5). There was significant increase in total lung proteins in the groups of rats which were treated with paraquat, paraquat and propranolol, or paraquat and theophylline compared to their respective controls. Lung cyclic AMP concentrations were significantly increased in rats which received paraquat (four-fold increase), or paraquat and theophylline (ten-fold increase) (Figure 18).

Lung cyclic GMP concentrations were significantly increased in the rats which received paraquat (eight-fold increase), paraquat and propranolol (eight-fold increase), and paraquat and theophylline (three-fold increase) compared to controls (Figure 18). Lung concentrations of paraquat in the rats which received paraquat, paraquat and propranolol, or paraquat and theophylline were not significantly different but lung propranolol concentrations in rats which received paraquat and propranolol were significantly higher while theophylline concentrations were significantly lower in rats treated with paraquat and theophylline compared to controls (Figure 19).

Rat Chronic In Vivo Experiment IV: The d- and l- forms of propranolol were given with paraquat in this experiment. Two rats died from the group treated with paraquat and l-propranolol. Lung cyclic AMP levels in rats treated with paraquat and d-propranolol showed about three-fold increase while the paraquat and l-propranolol treated rats showed a significant reduction. The cyclic AMP levels in the paraquat and d-propranolol treated rats were also significantly higher compared to the paraquat treated group (Figure 20).

Lung cyclic GMP concentrations were significantly increased in rats treated with paraquat and d-propranolol compared to their controls but were not different from rats treated with only paraquat. Lung cyclic GMP levels in paraquat and l-propranolol treated rats were not significantly different compared to l-propranolol treated rats (Figure 20).

Rat Acute In Vivo Experiment I

In this experiment, two groups of rats received either saline or paraquat 20 mg cation (27.6 mg) per Kg body weight. Lung cyclic GMP was elevated with a peak at 30 min after paraquat injection, the level dropping to the control level, followed by a slight rise at about 8 hr and then a gradual fall to the control value (Figure 21).

The lung cyclic AMP also showed a rise, the peak level occurring at 1 1/2 hr followed by a fall to the control value which was maintained over the 24 hr period of the experiment. There was a rapid uptake of paraquat into the lungs with concentration peak (10 nm/mg protein) at 2 hr, then a gradual fall to about 6 ng/mg protein over the rest of the period of the experiment (Figure 21).

Rat Acute In Vivo Experiment II

In this experiment, the effect of the adrenergic and cholinergic systems on lung cyclic nucleotides was tested.

Lung cyclic AMP values in the rats pretreated with reserpine were not significantly different from those of saline pretreated rats (Table 6). Lung cyclic GMP results were inconsistent in the reserpine pretreated rats compared to controls. The lung cyclic AMP values in the rats pretreated with atropine were all significantly lower compared

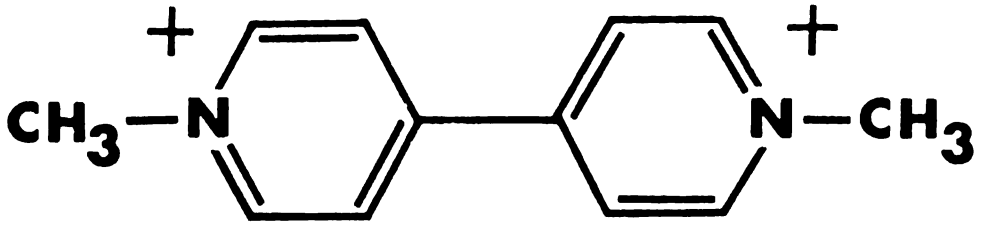
to saline pretreated controls (Table 6). Lung cyclic GMP levels in atropine pretreated rats were only significantly different in rats killed at 2 hr after paraquat injection (Table 6).

Effect of Paraquat on Cyclic Nucleotides of Rat Liver, Spleen, Kidney and Thymus

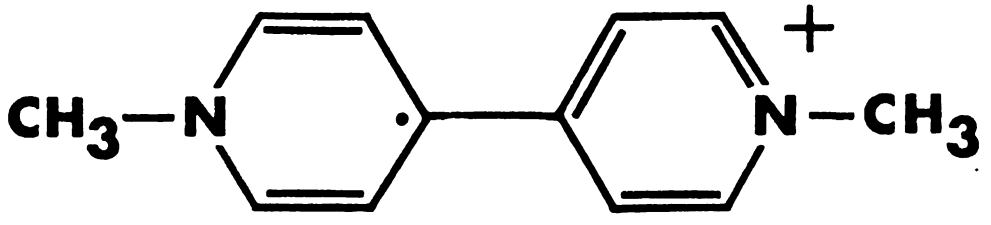
In this experiment it was observed that the cyclic nucleotides, cyclic AMP and cyclic GMP were significantly higher in lungs of rats treated with paraquat for 8 days while levels in the organs studied--namely; liver, spleen, thymus, and kidney were not significantly different from their controls (Table 7).

Figure 3. Structure of paraquat.

PARAQUAT



(oxidized)



(reduced)

Figure 3

Figure 4. Rat isolated lung perfusion experiment showing percent weight change in the lung on perfusing with paraquat, propranolol, paraquat and propranolol, paraquat and mannitol, histamine, Bovine-serum albumin-Kreb's solution (BSA). Values represent mean $\pm$ SEM.

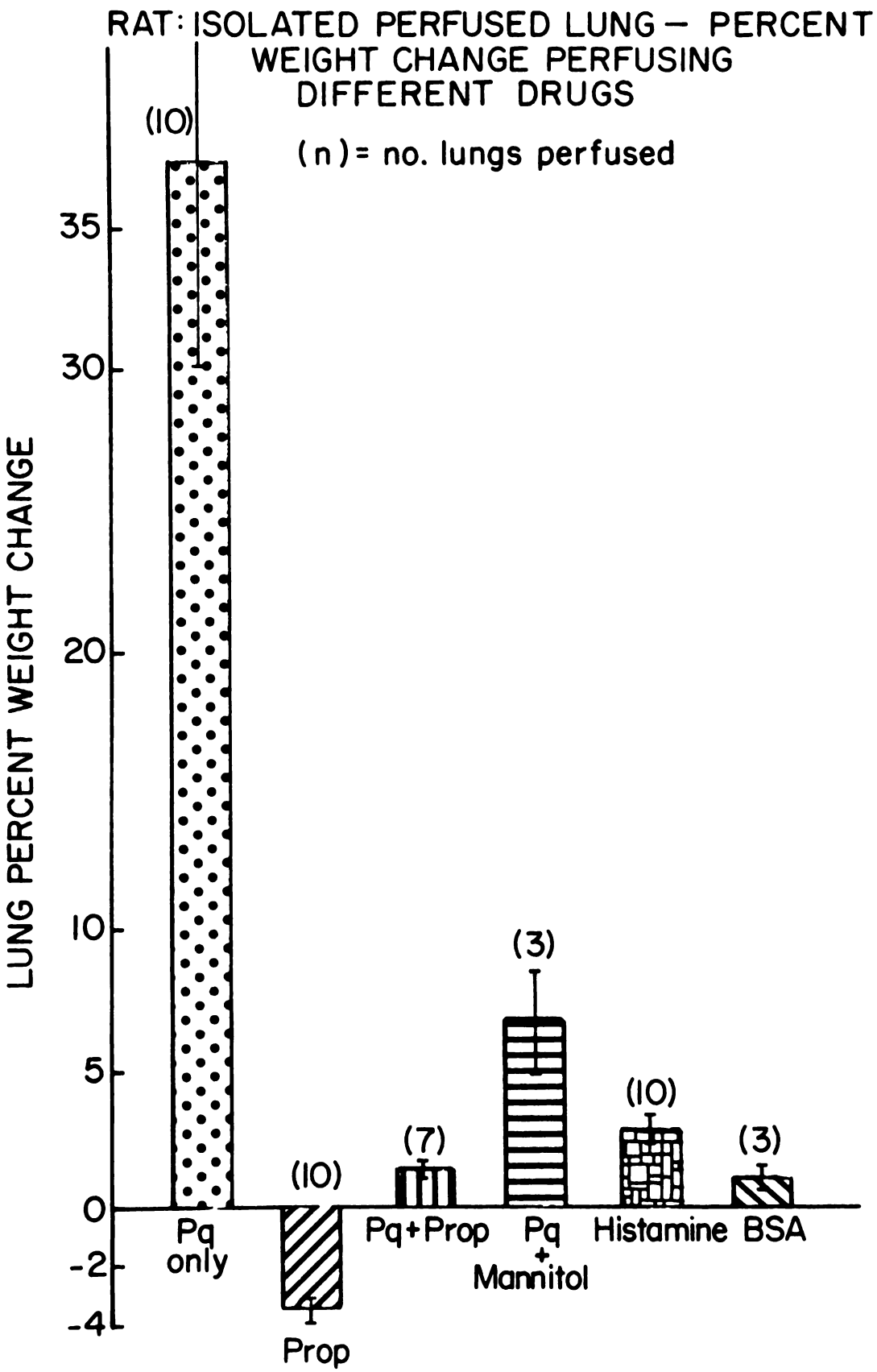

RAT: ISOLATED PERFUSED LUNG — PERCENT WEIGHT CHANGE PERFUSING DIFFERENT DRUGS
(n) = no. lungs perfused
LUNG PERCENT WEIGHT CHANGE
35
30
20
10
5
0
-2
-4
(10)
(10)
(7)
(3)
(10)
(3)
Pq only
Prop
Pq+Prop
Pq + Mannitol
Histamine
BSA

Figure 5. Rat isolated lung perfusion experiment--showing glucose utilization with time. Values represent mean ± SEM. The slopes of the glucose disappearance curves were significantly different ($p < 0.05$).

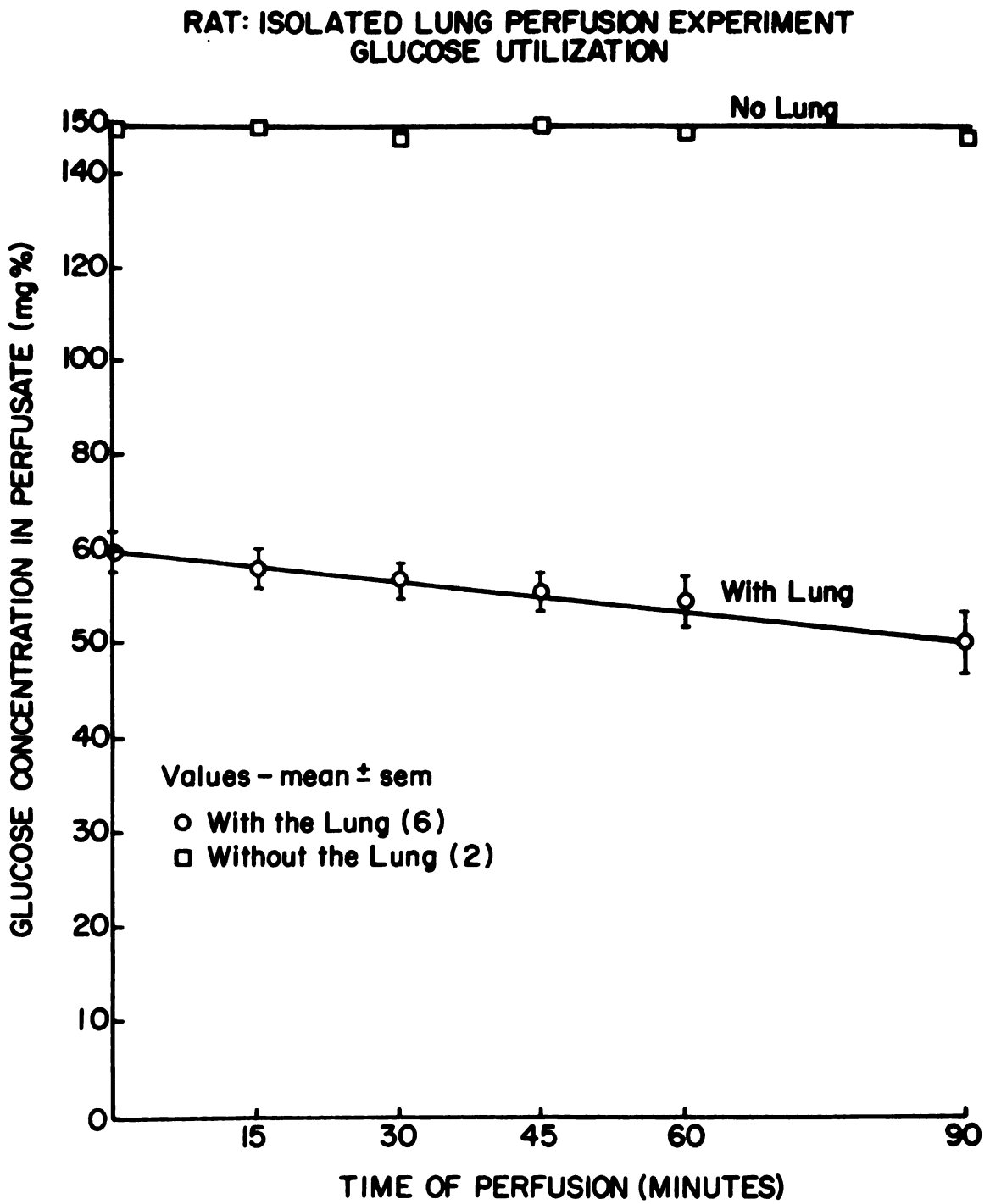

RAT: ISOLATED LUNG PERFUSION EXPERIMENT
GLUCOSE UTILIZATION
No Lung
With Lung
Values – mean ± sem
With the Lung (6)
Without the Lung (2)
GLUCOSE CONCENTRATION IN PERFUSATE (mg %)
TIME OF PERFUSION (MINUTES)
0
10
20
30
40
50
60
80
100
120
140
150
15
30
45
60
90

Figure 6. Rat isolated lung perfusion experiment--showing cyclic AMP changes after perfusing with isoproterenol, paraquat, paraquat and propranolol and BSA solution. Values represent mean ± SEM.

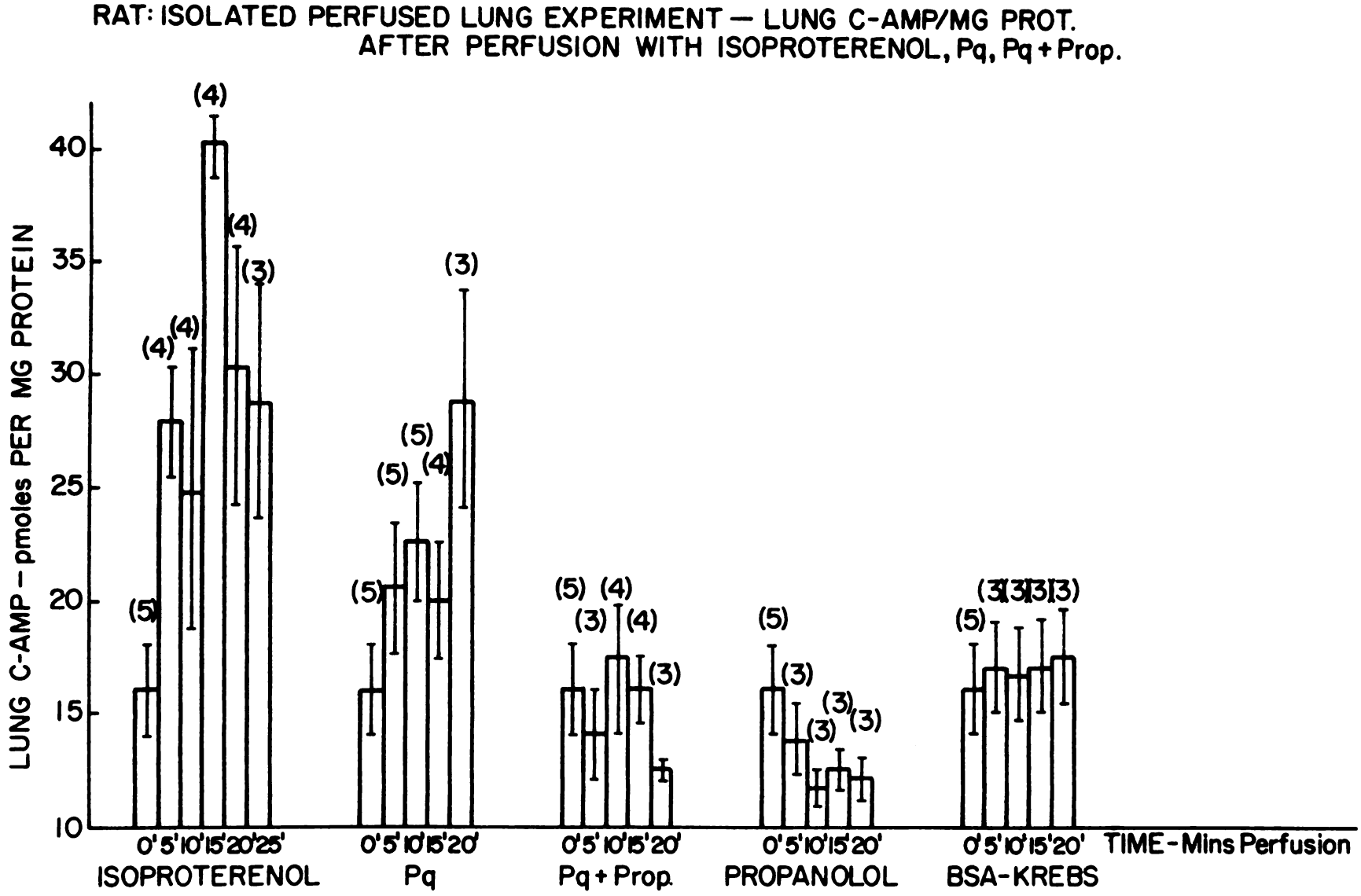

RAT: ISOLATED PERFUSED LUNG EXPERIMENT — LUNG C-AMP/MG PROT.
AFTER PERFUSION WITH ISOPROTERENOL, Pq, Pq + Prop.
LUNG C-AMP – pmoles PER MG PROTEIN
10
15
20
25
30
35
40
0'5'10'15'20'25'
ISOPROTERENOL
0'5'10'15'20'
Pq
0'5'10'15'20'
Pq + Prop.
0'5'10'15'20'
PROPANOLOL
0'5'10'15'20'
BSA-KREBS
TIME - Mins Perfusion

Figure 7. Effect of paraquat on body weight of rats compared to saline treated rats. Rats were treated daily with paraquat (5 mg/kg) ip or 0.9% NaCl (0.05 ml/kg) ip daily and weighed. Each point represents the mean weight $\pm$ SEM for 25 rats.

RAT: CHRONIC IN VIVO EXPERIMENT - (I) -
CHANGE IN BODY WEIGHT IN PARAQUAT
AND SALINE TREATED RATS

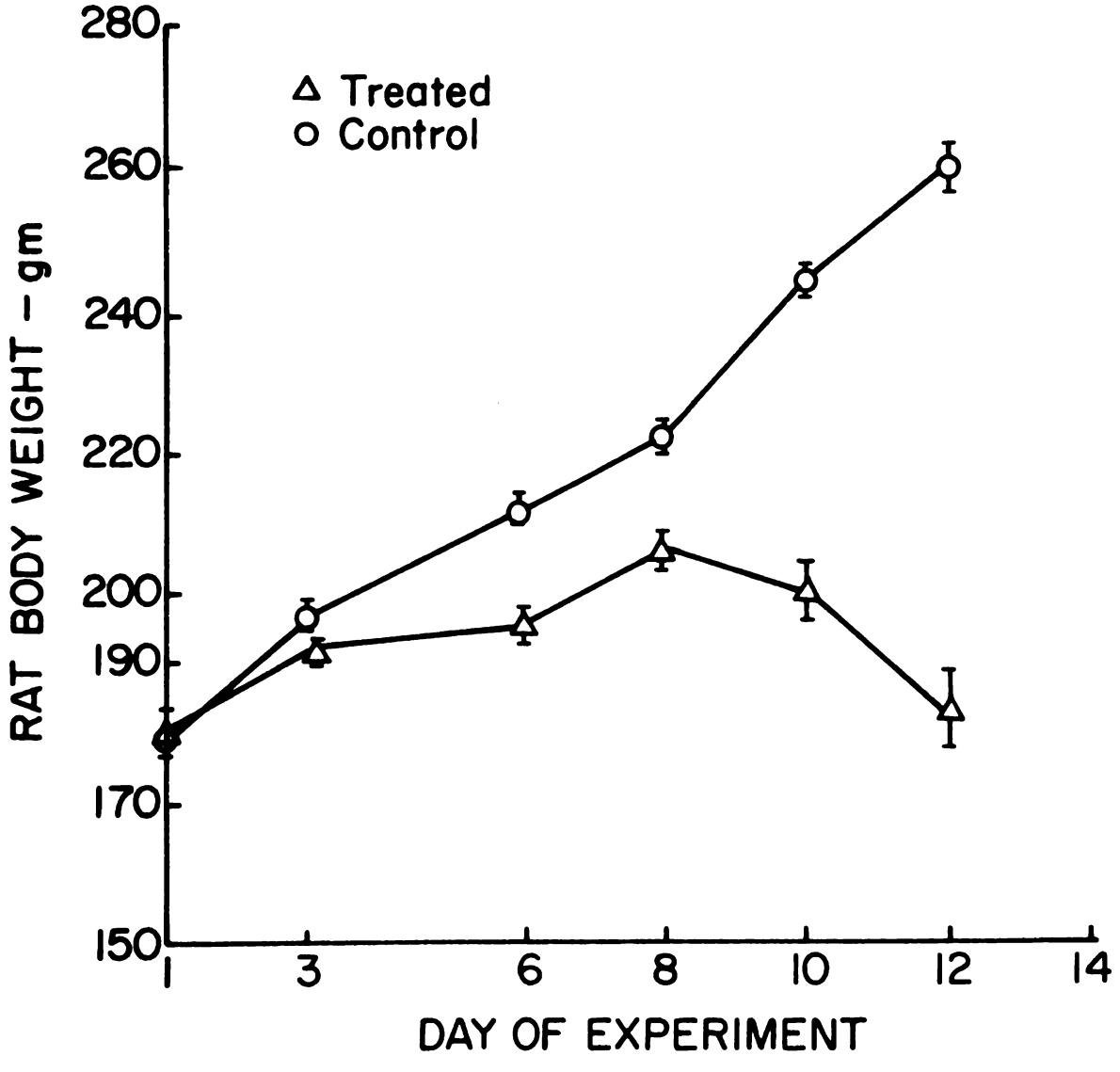


Figure 8. Effect of paraquat on lung total proteins compared to saline treated rats. Rats were treated with paraquat (5 mg/kg) ip or 0.9% NaCl (0.05 ml/10g) ip daily. Five rats from each group were killed on the 3rd, 6th, 8th, 10th and 12th days and total proteins per lung determined. Each point represents the mean total lung proteins ± SEM for 5 rats.

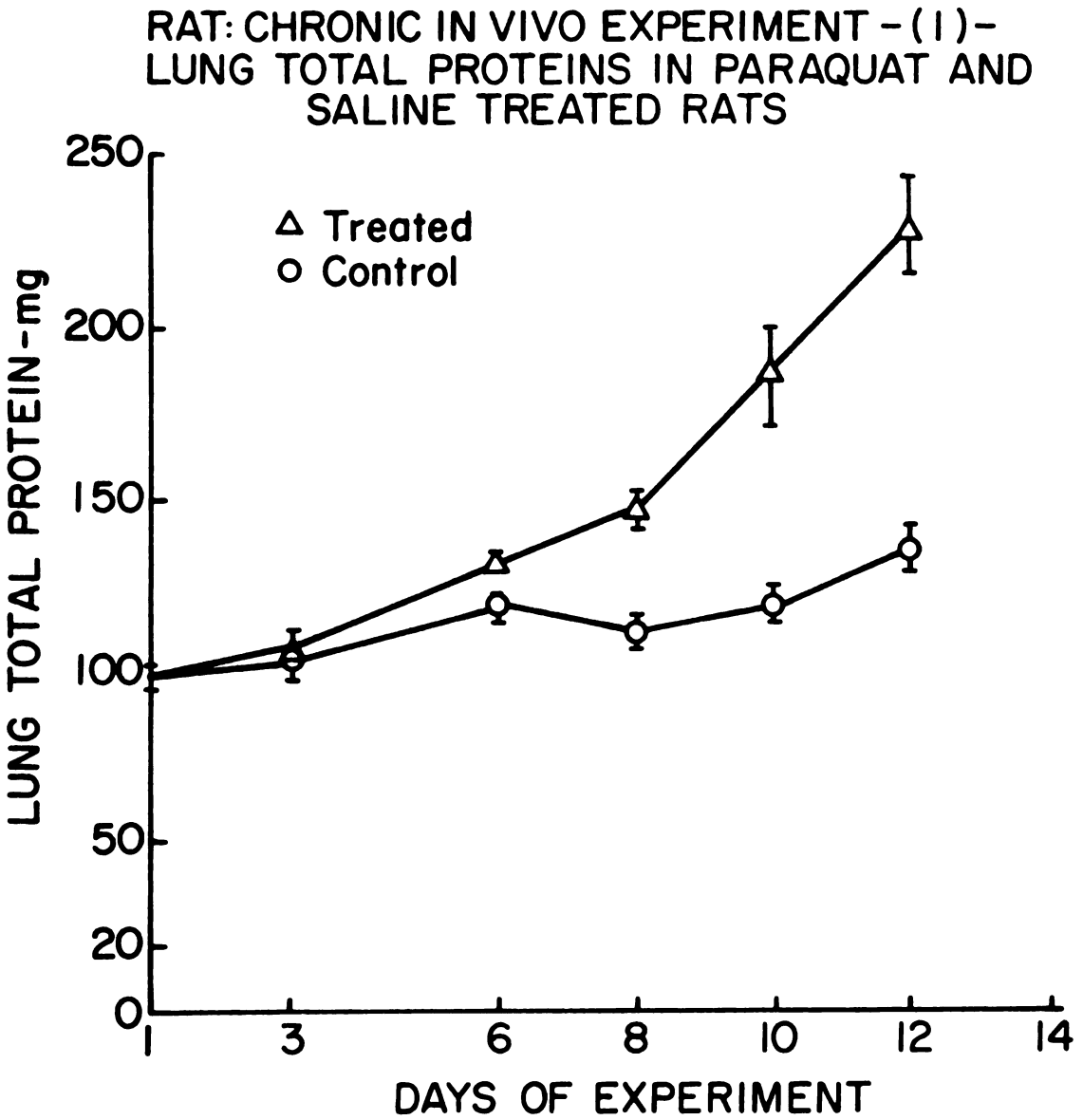

RAT: CHRONIC IN VIVO EXPERIMENT - (I) -
LUNG TOTAL PROTEINS IN PARAQUAT AND
SALINE TREATED RATS
Δ Treated
O Control
LUNG TOTAL PROTEIN-mg
250
200
150
100
50
20
0
1
3
6
8
10
12
14
DAYS OF EXPERIMENT

Figure 9. Effect of paraquat on lung c-AMP compared to saline treated rats. Rats were treated with paraquat (5 mg/kg) ip or 0.9% NaCl (0.05 ml/10g) ip daily. Five rats from each group were killed on the 3rd, 6th, 8th, 10th and 12th days and lung c-AMP and paraquat concentrations were determined. Each point represents the mean c-AMP or paraquat concentration $\pm$ SEM for 5 rats.

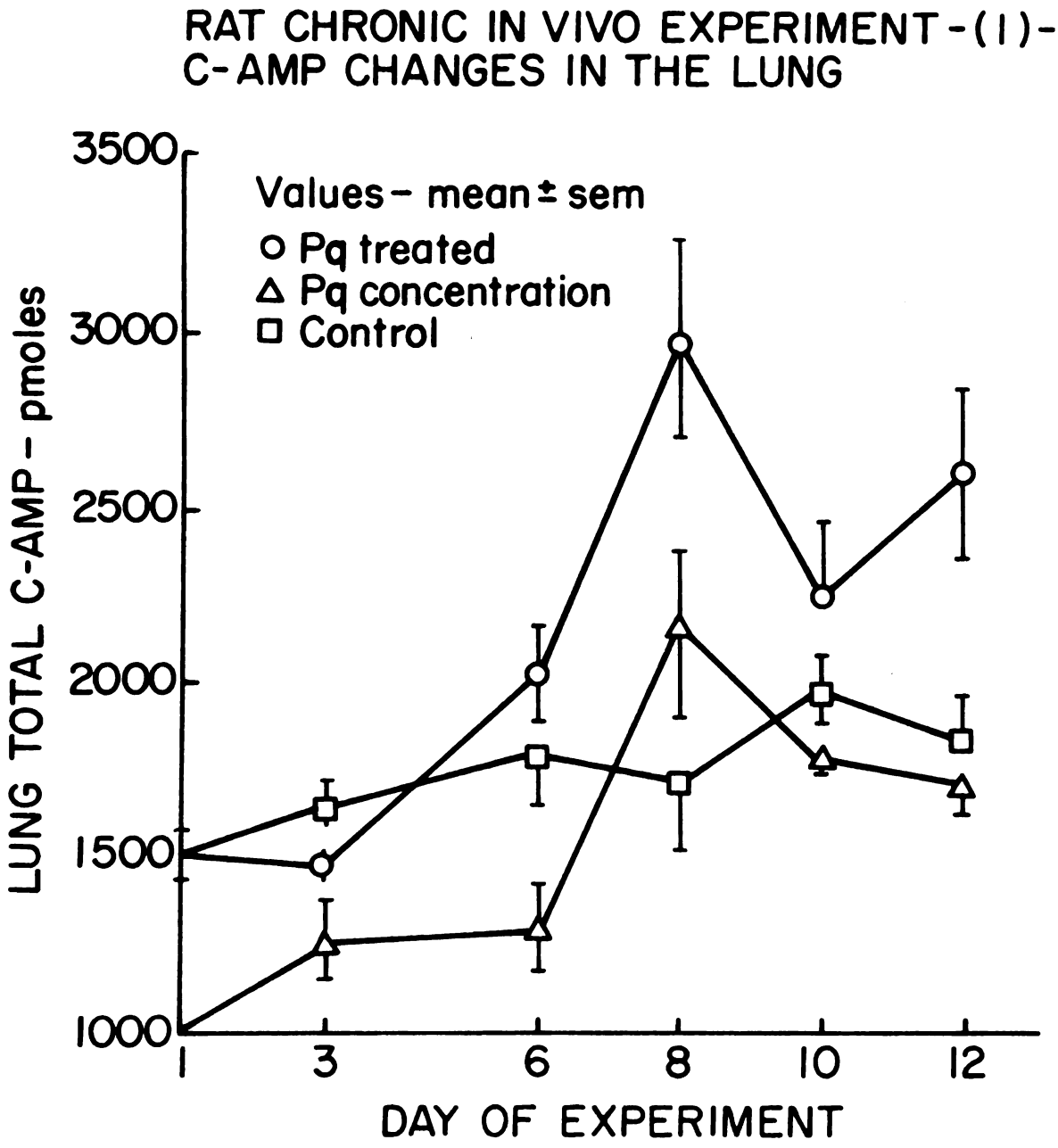

RAT CHRONIC IN VIVO EXPERIMENT-(I)-
C-AMP CHANGES IN THE LUNG
Values- mean ± sem
Pq treated
Pq concentration
Control
LUNG TOTAL C-AMP- pmoles
3500
3000
2500
2000
1500
1000
1
3
6
8
10
12
DAY OF EXPERIMENT

Figure 10. Effect of paraquat on lung c-GMP compared to saline treated rats. Rats were treated with paraquat (5 mg/kg) ip or 0.9% NaCl (0.05 ml/10g) ip daily. Five rats from each group were killed on the 3rd, 6th, 10th and 12th days and lung c-GMP and paraquat concentrations were determined. Each point represents the mean c-GMP or paraquat concentration $\pm$ SEM for 5 rats.

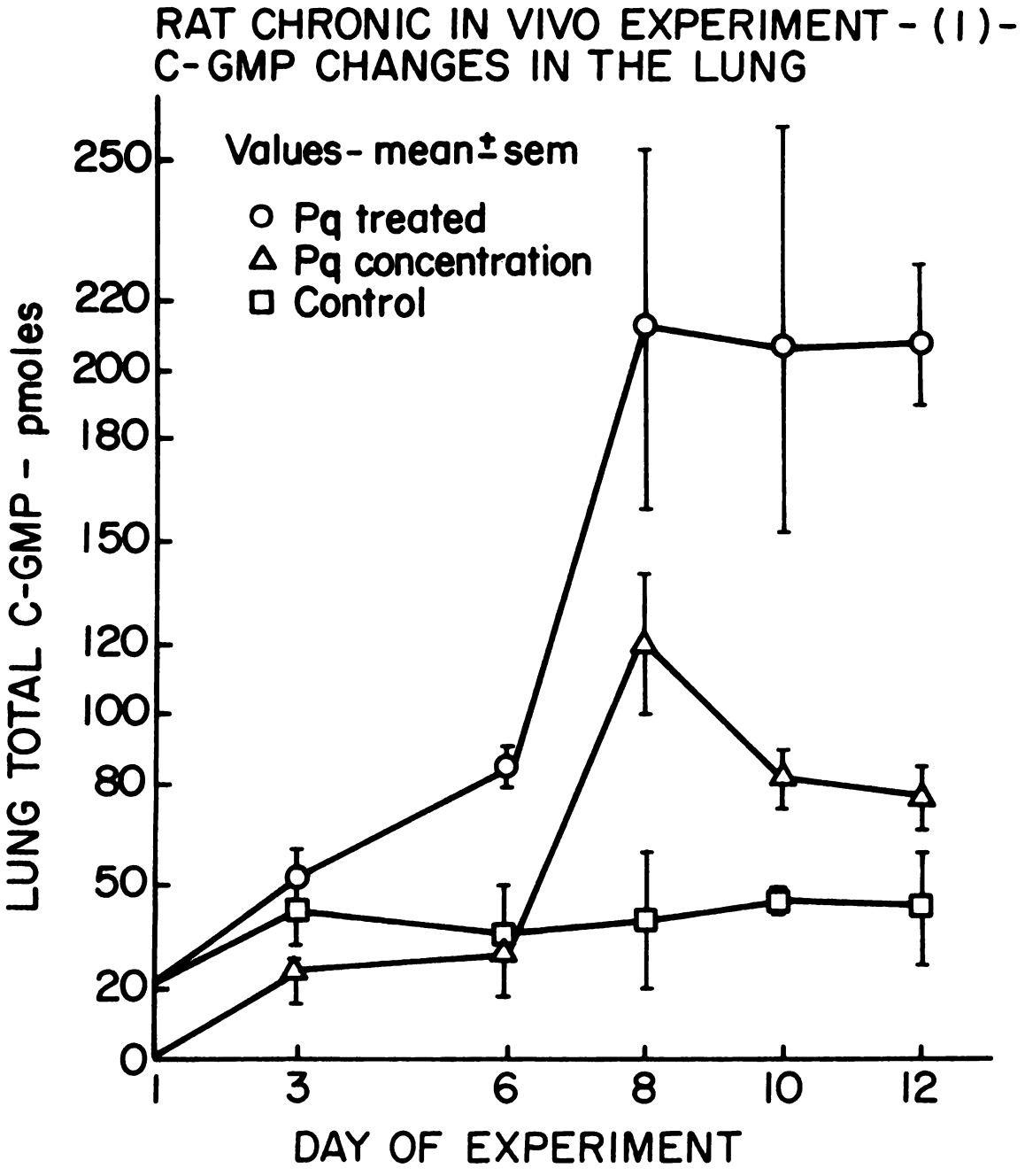

RAT CHRONIC IN VIVO EXPERIMENT - (I) -
C-GMP CHANGES IN THE LUNG
Values- mean± sem
Pq treated
Pq concentration
Control
LUNG TOTAL C-GMP - pmoles
250
220
200
180
150
120
100
80
50
20
0
1
3
6
8
10
12
DAY OF EXPERIMENT

Figure 11. Effects of paraquat, paraquat and propranolol, and paraquat and theophylline on rat body weight compared to rats treated with saline, propranolol or theophylline. Rats were weighed daily and treated with saline (0.05 ml/10g), paraquat (5 mg/kg ip), propranolol (5 mg/kg sc 3 x daily), theophylline (10 mg/kg ip) or combinations of either paraquat and propranolol, or paraquat and theophylline in corresponding doses. Each point represents the mean weight $\pm$ SEM.

RAT CHRONIC IN VIVO EXPERIMENT-(2)-
RAT BODY WEIGHT CHANGES IN DIFFERENT GROUPS

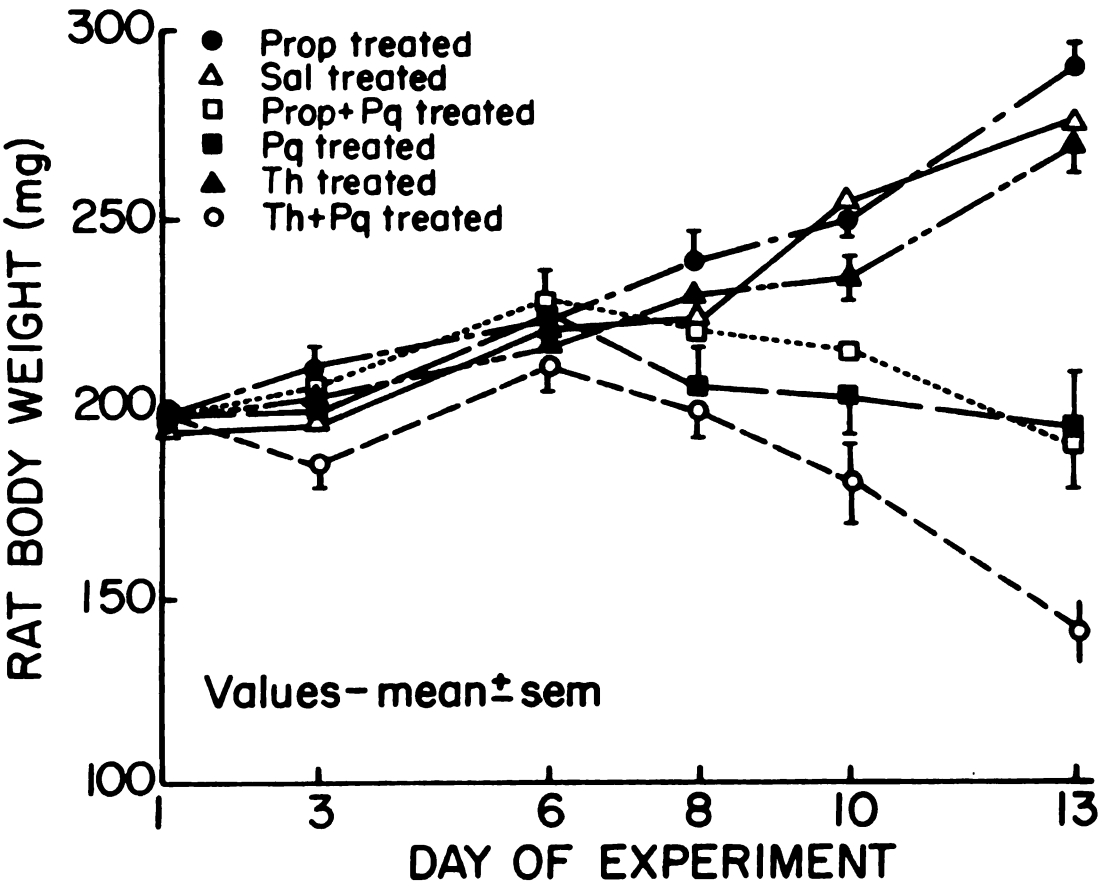


Figure 12. Effects of paraquat, propranolol, and paraquat and propranolol on rat lung c-AMP. Rats were treated daily with 0.9% NaCl (0.05 ml/10g), paraquat (5 mg/kg ip), propranolol (5 mg/kg sc) or paraquat and propranolol in corresponding doses. Five rats from each group were killed on the 3rd, 6th, 8th, 10th and 13th days and lung c-AMP concentrations determined. Each point represents the mean c-AMP concentration $\pm$ SEM for 5 rats.

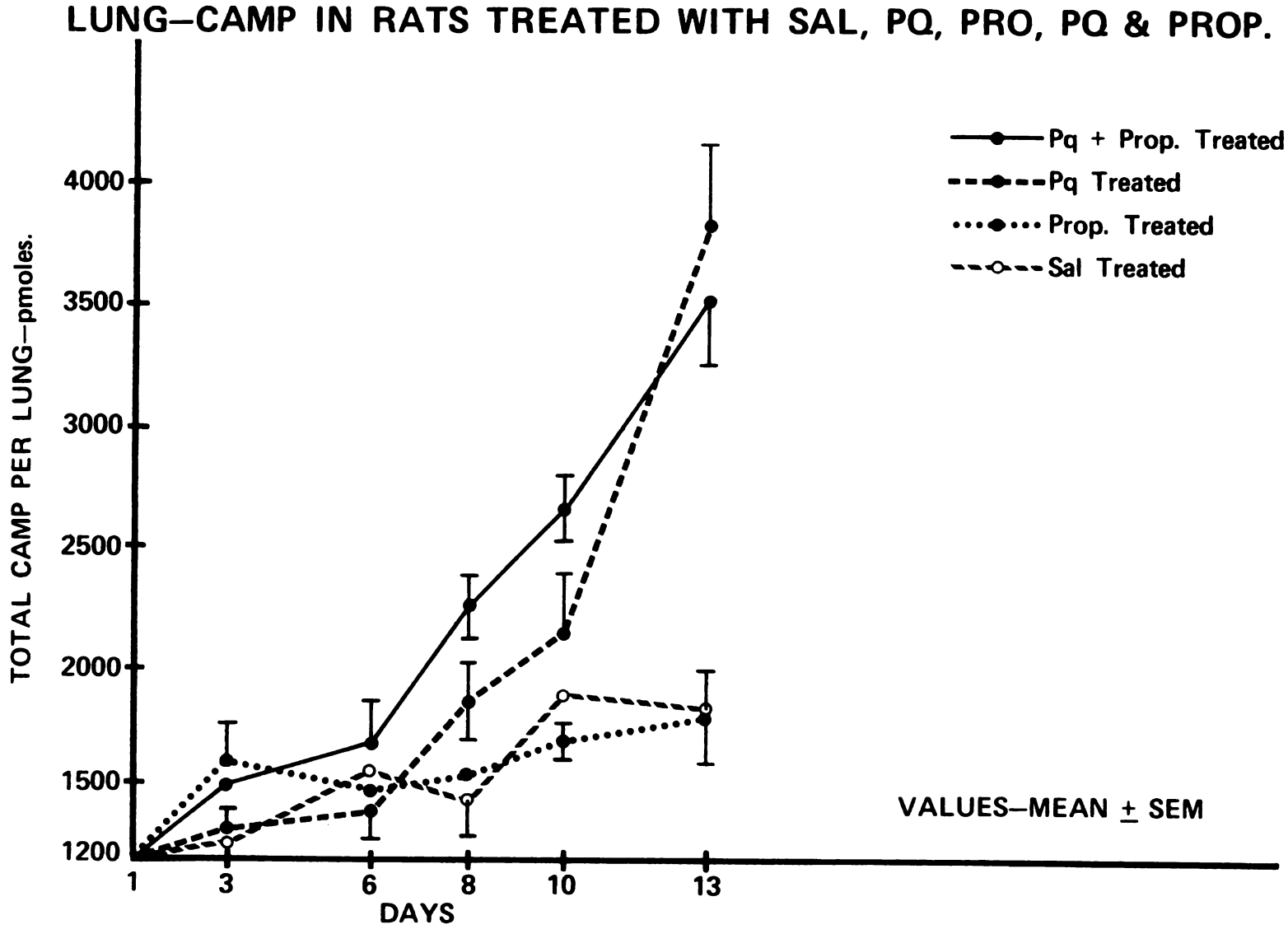

LUNG–CAMP IN RATS TREATED WITH SAL, PQ, PRO, PQ & PROP.
TOTAL CAMP PER LUNG–pmoles.
4000
3500
3000
2500
2000
1500
1200
1
3
6
8
10
13
DAYS
Pq + Prop. Treated
Pq Treated
Prop. Treated
Sal Treated
VALUES–MEAN ± SEM

Figure 13. Effects of paraquat, theophylline, and paraquat and theophylline on rat lung c-AMP. Rats were treated daily with saline (0.05 ml/10g ip) paraquat (5 mg/kg ip), theophylline in corresponding doses. Five rats from each group were killed on the 3rd, 6th, 8th, 10th and 13th days and the lung c-AMP determined. Each point represents mean total lung c-AMP for 5 rats.

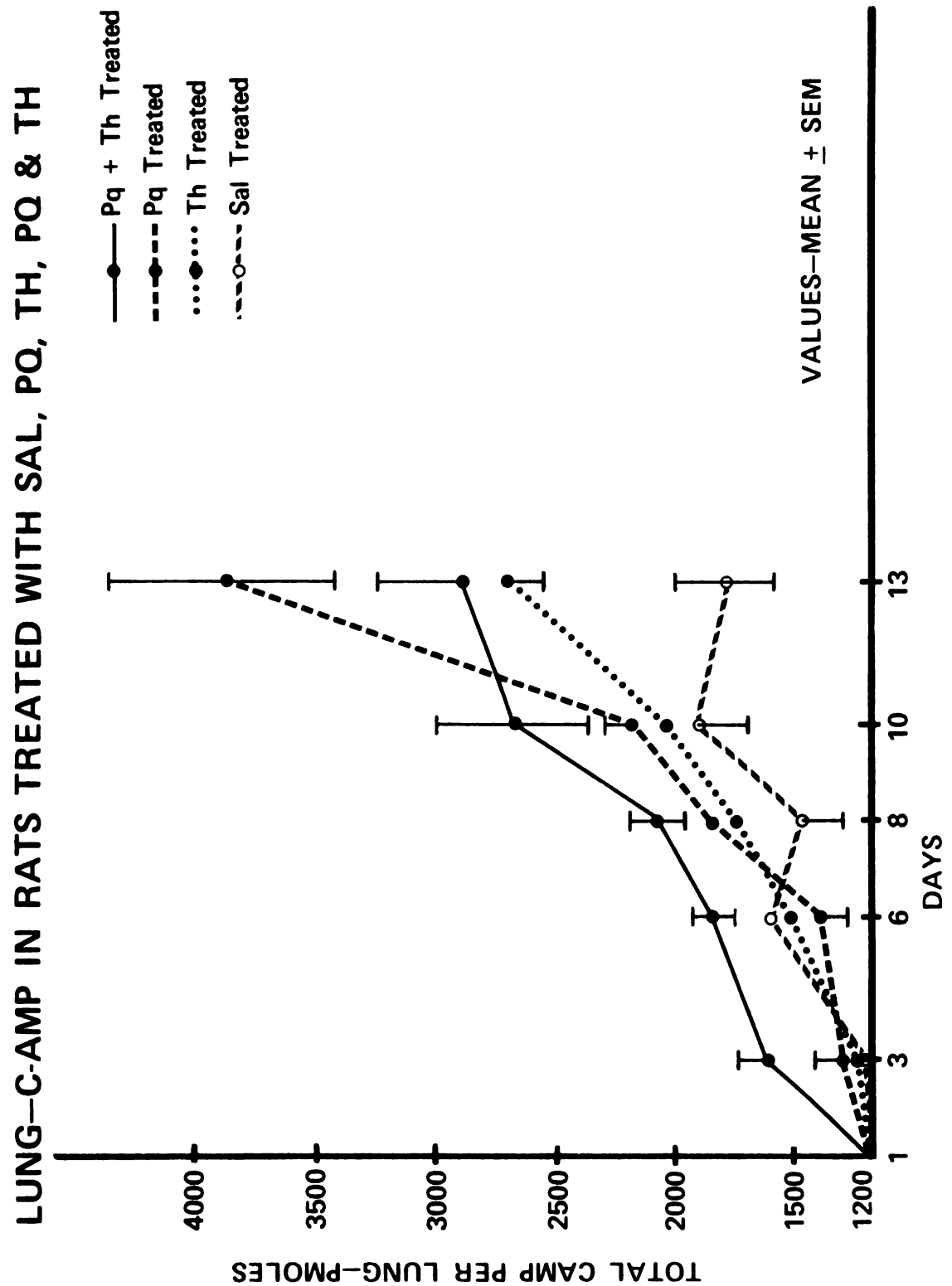

LUNG—C-AMP IN RATS TREATED WITH SAL, PQ, TH, PQ & TH
Pq + Th Treated
Pq Treated
Th Treated
Sal Treated
VALUES—MEAN ± SEM
TOTAL CAMP PER LUNG—PMOLES
4000
3500
3000
2500
2000
1500
1200
1
3
6
8
10
13
DAYS

Figure 14. Effect of paraquat, propranolol, and paraquat and propranolol on lung c-GMP. Rats were treated daily with saline (0.05 ml/10g), paraquat (5 mg/kg ip), propranolol (5 mg/kg sc 3x) or paraquat and propranolol in corresponding doses. Five rats from each group were killed on the 3rd, 6th, 8th, 10th and 13th days and the lung c-GMP determined. Each point represents the mean total c-GMP $\pm$ SEM for 5 rats.

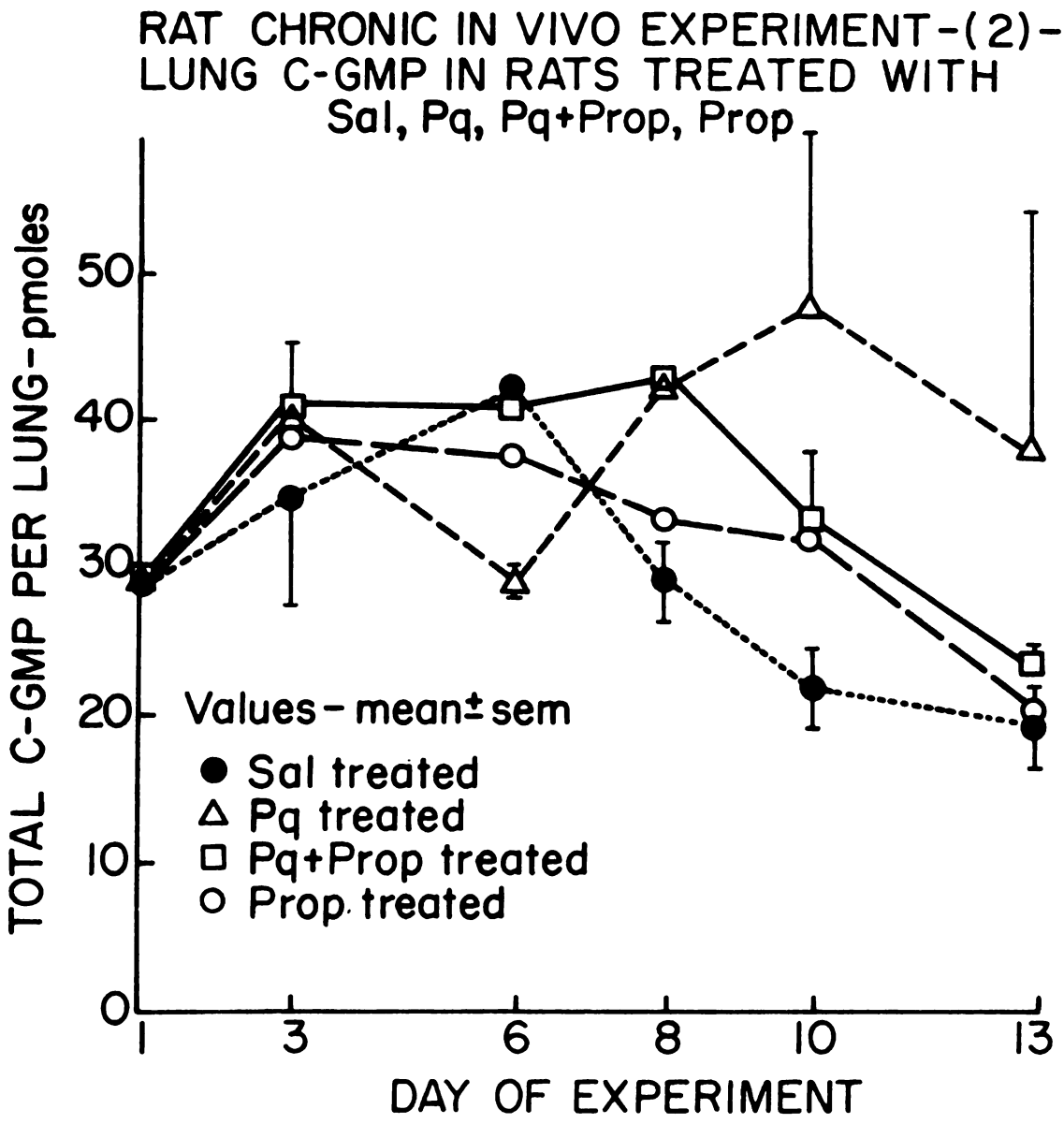

RAT CHRONIC IN VIVO EXPERIMENT-(2)-
LUNG C-GMP IN RATS TREATED WITH
Sal, Pq, Pq+Prop, Prop
TOTAL C-GMP PER LUNG-pmoles
0
10
20
30
40
50
1
3
6
8
10
13
DAY OF EXPERIMENT
Values-mean± sem
Sal treated
Pq treated
Pq+Prop treated
Prop. treated

Figure 15. Effect of paraquat, theophylline and paraquat and theophylline on rat lung c-GMP. Rats were treated with saline (0.05 ml/10g ip), paraquat (5 mg/kg ip), theophylline (10 mg/kg ip) or paraquat + theophylline in corresponding doses. Five rats from each group were killed on the 3rd, 6th, 8th, 10th and 13th days and lung c-GMP determined. Each point represents mean total c-GMP±SEM for 5 rats.

RAT: CHRONIC IN VIVO EXPERIMENT-(2)-
LUNG C-GMP CHANGES IN RATS TREATED
WITH SALINE, Pq, Pq+Th, Th

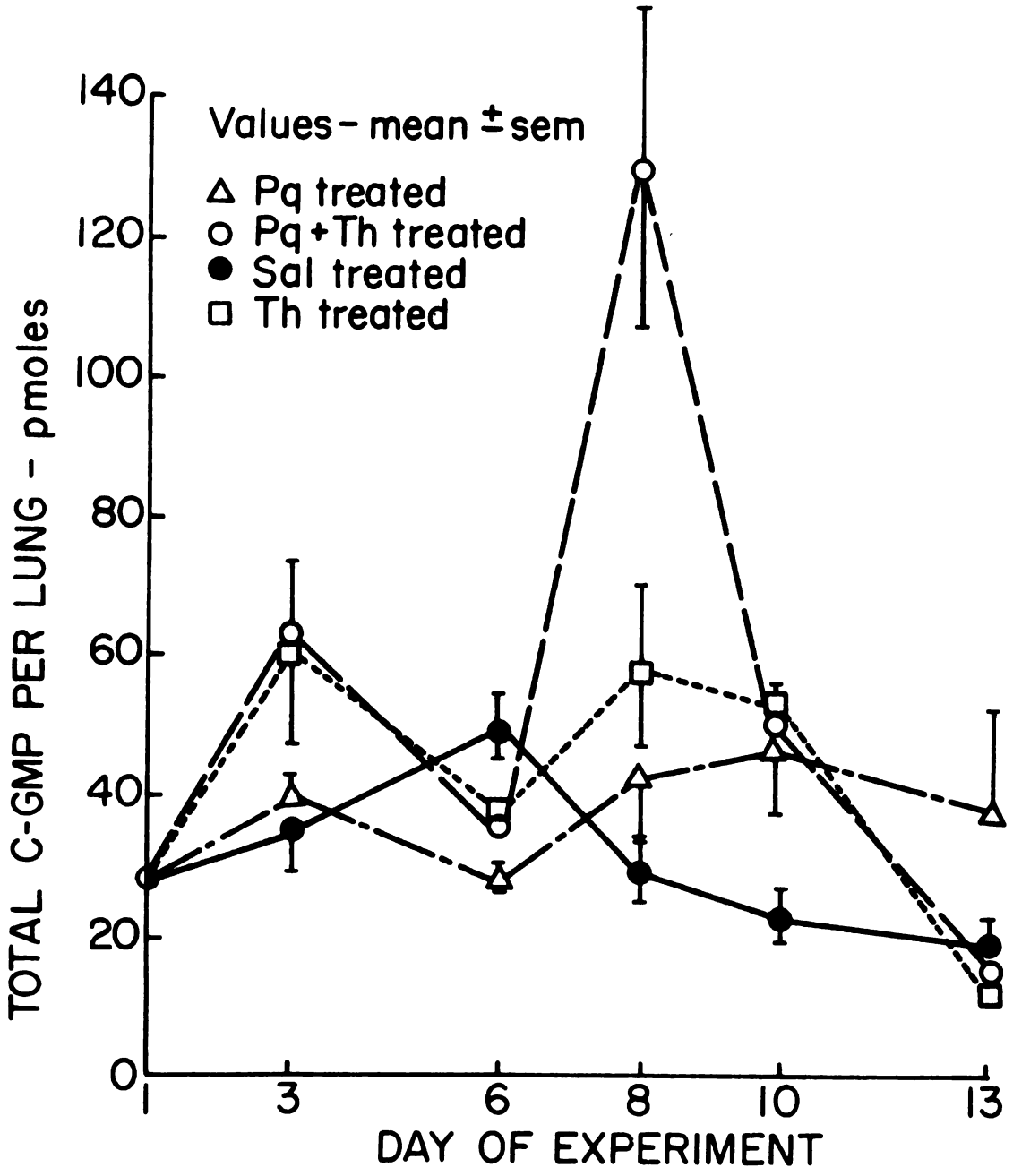


Figure 16. Long concentrations of paraquat and propranolol in groups of rats treated with paraquat (Group B), paraquat + propranolol (Group C), propranolol (Group D), or paraquat + theophylline (Group F). Five rats from each group were killed on the 3rd, 6th, 8th, 10th and 13th days and lung concengrations of paraquat and propranolol determined. Each point represents mean total concentration of paraquat or propranolol $\pm$ SEM for 5 rats. (Group F included for comparison.)

RAT: CHRONIC IN VIVO EXPERIMENT-(2)-
CONCENTRATION OF Pq AND Prop IN GROUPS
B, C, D, AND F

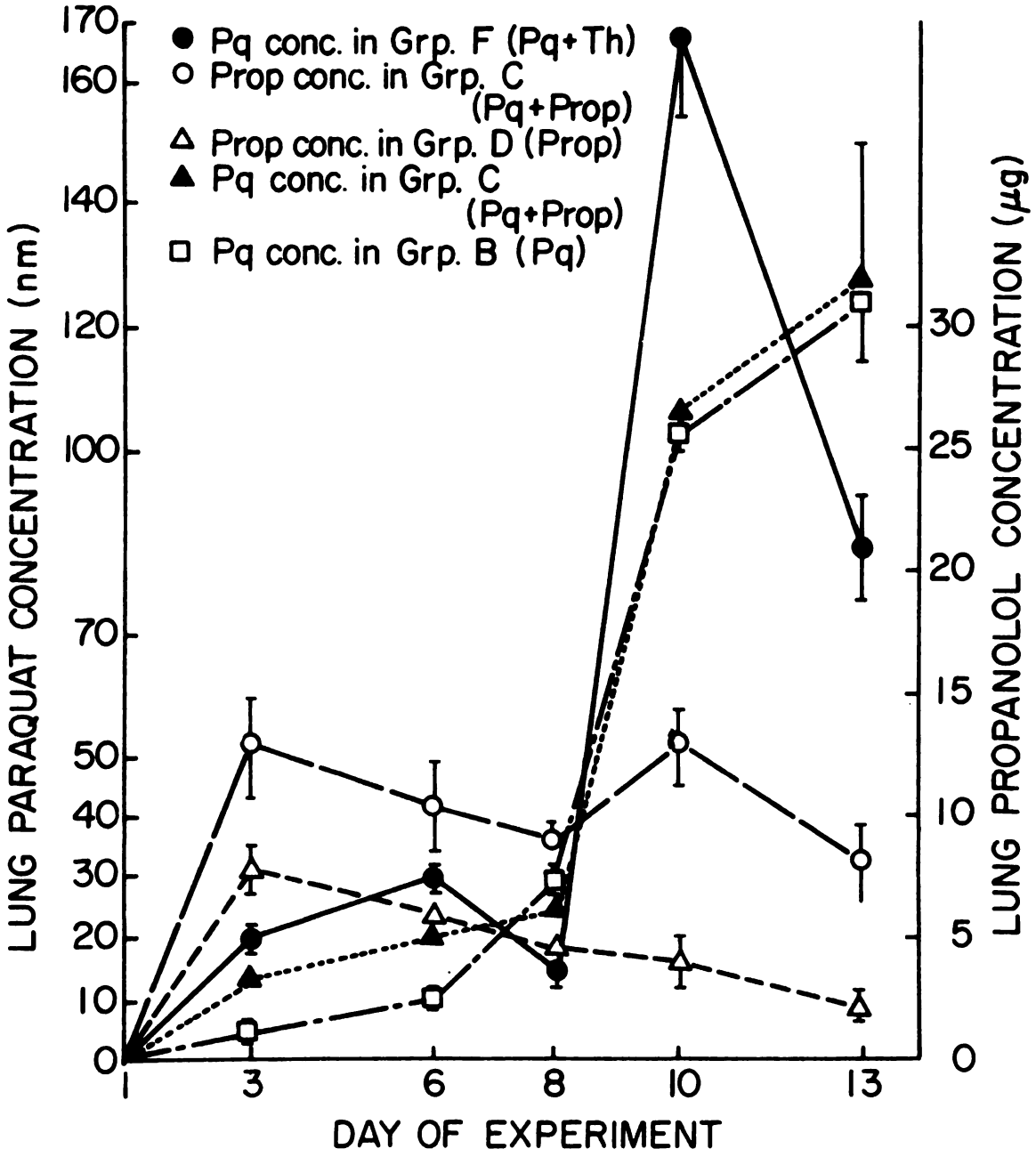


Figure 17. Lung concentrations of paraquat and theophylline in groups of rats treated with paraquat (Group B), theophylline (Group E) or paraquat and theophylline (Group F). Five rats from each group were killed on the 3rd, 6th, 8th, 10th and 13th days and lung concentrations of paraquat and theophylline determined. Each point represents mean total concentration of paraquat or theophylline $\pm$ SEM for 5 rats.

RAT: CHRONIC IN VIVO EXPERIMENT-(2)-
CONCENTRATION OF Pq AND Theo IN
GROUPS B, E, AND F

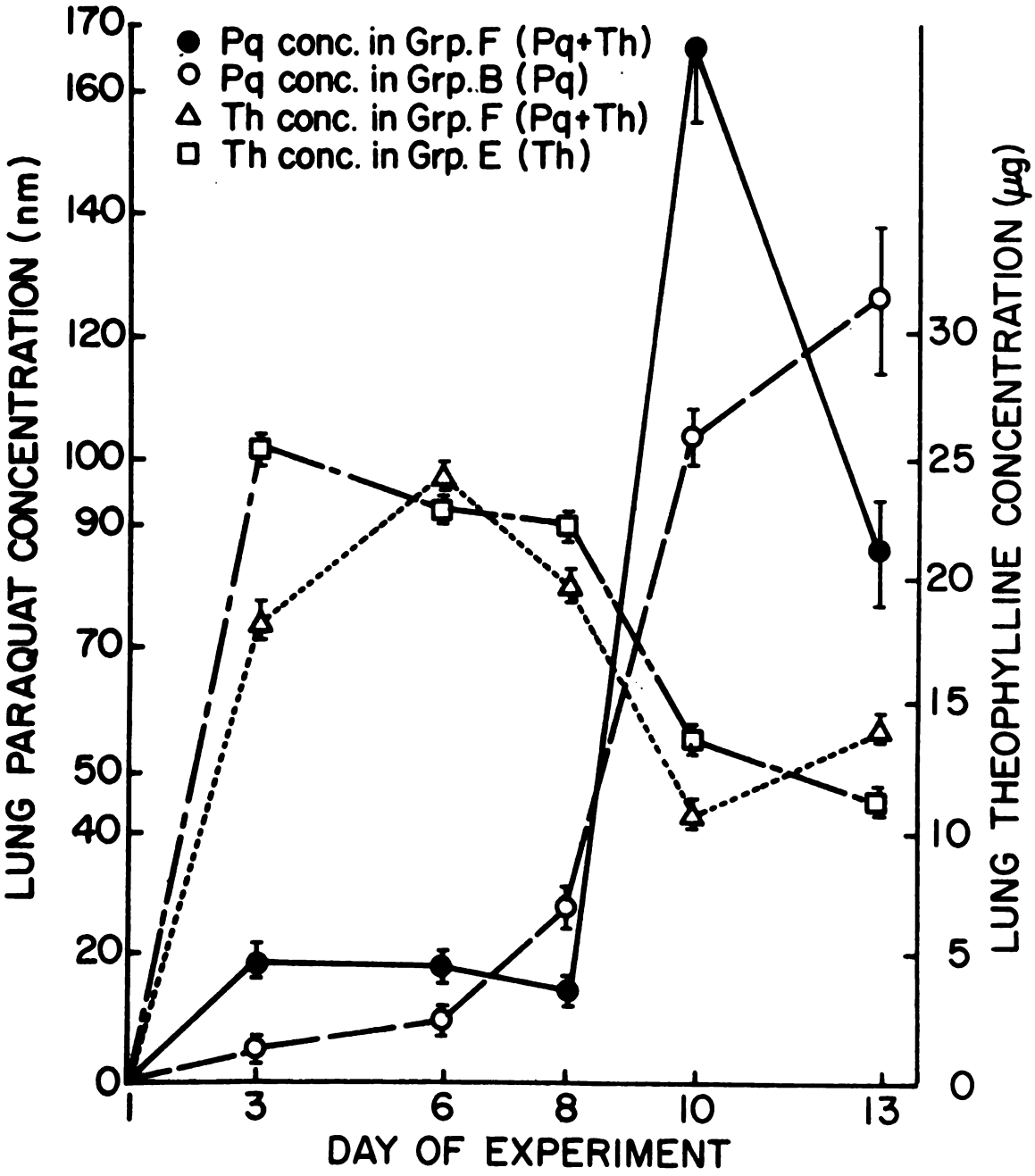
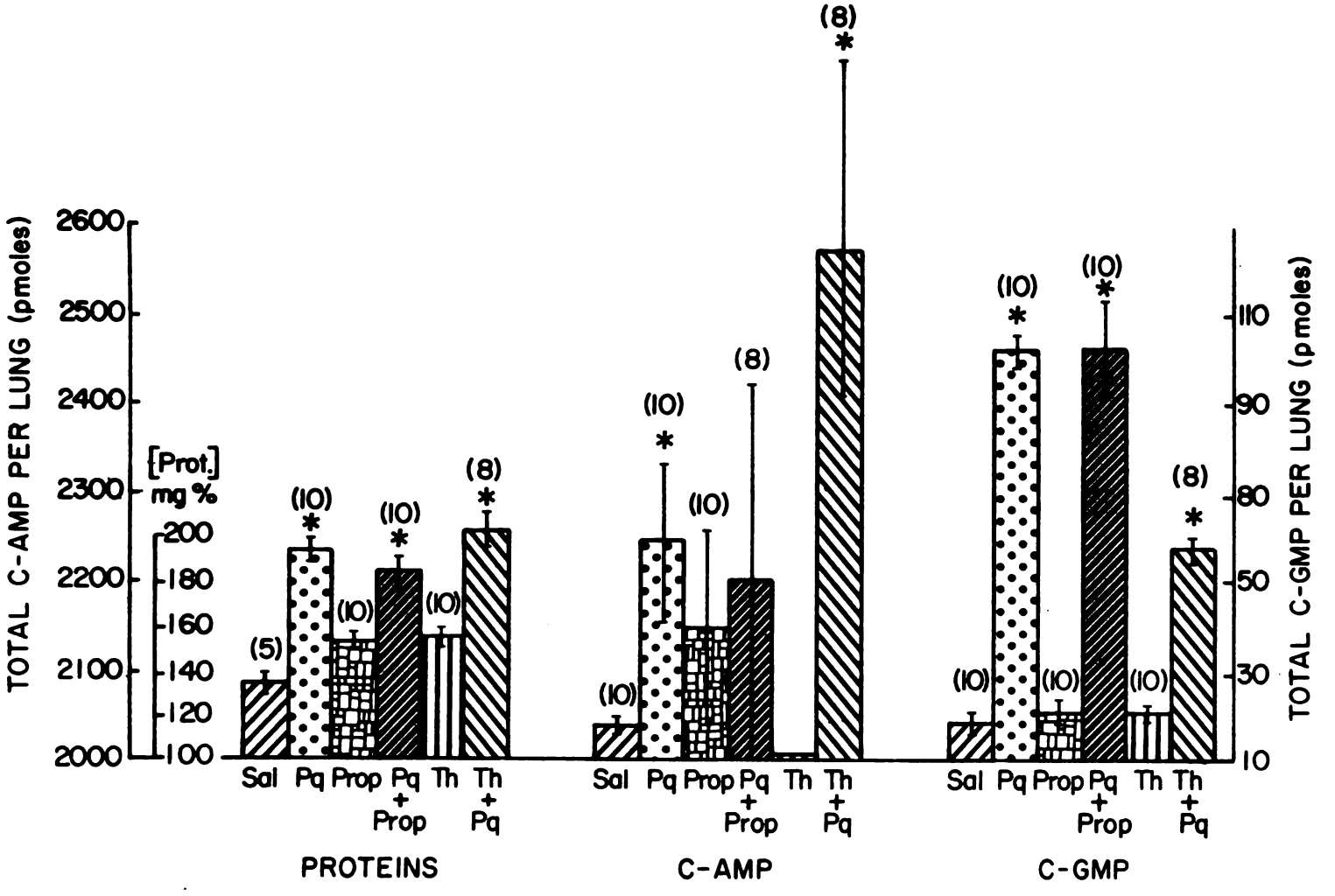


Figure 18. Effects of paraquat, propranolol, theophylline, paraquat and propranolol, and paraquat and theophylline on rat lung total proteins, total c-AMP and total c-GMP after 9 days treatment. Values represent mean ± SEM for 5 rats. Asterisk indicates values significantly different from controls ($p < 0.05$).

RAT: CHRONIC IN VIVO EXPERIMENT-(3)-LUNG TOTAL PROTEINS, C-AMP AND C-GMP CHANGES



* *Values significantly different from controls (P<0.05)*

Figure 19. Rat lung concentrations of paraquat, propranolol and theophylline after treatment with paraquat, paraquat and propranolol, paraquat and theophylline, propranolol or theophylline. Values represent mean + SEM. Asterisk indicates values which are significantly different from controls ($p < 0.05$).

RAT: CHRONIC IN VIVO EXPERIMENT (3)

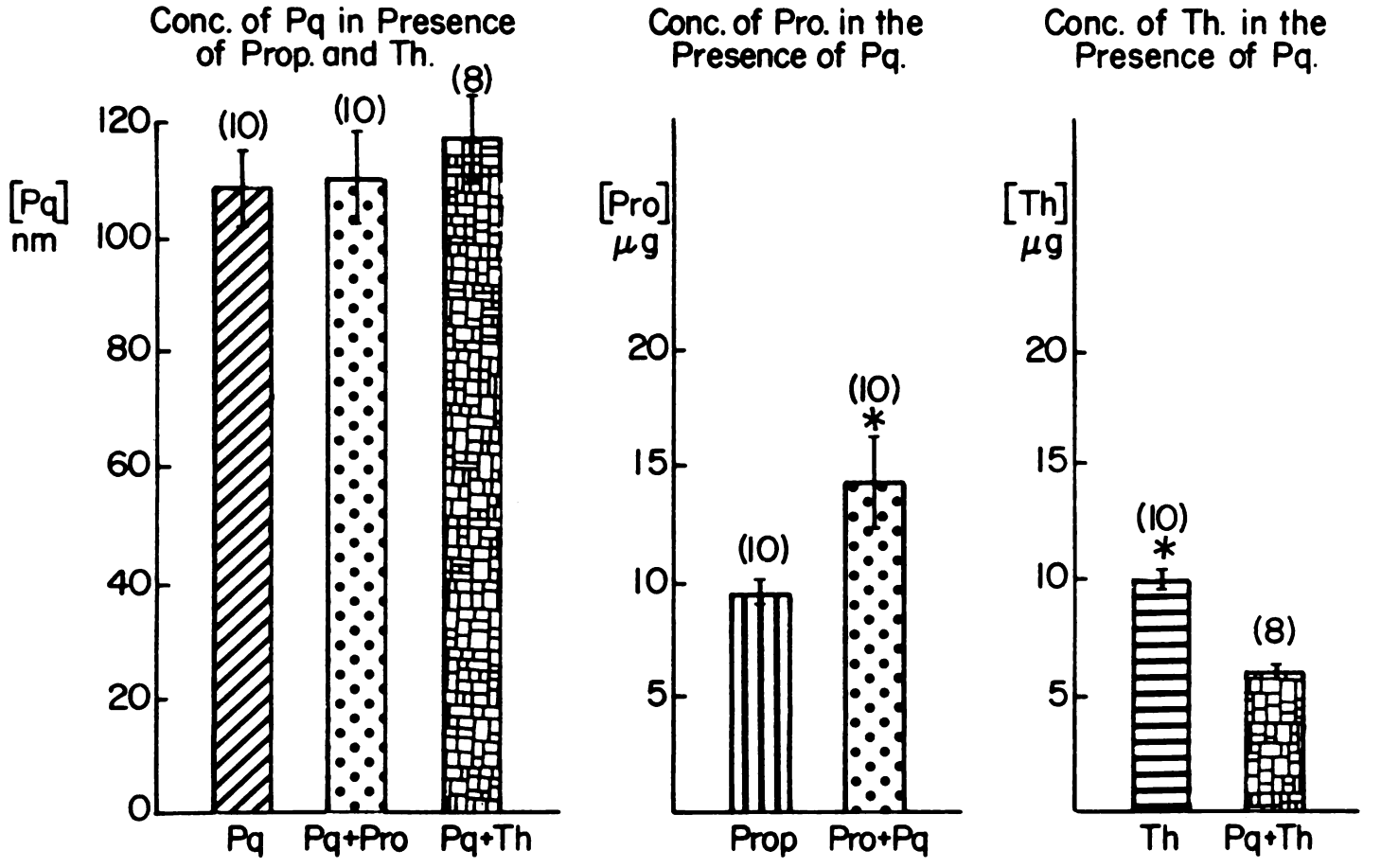
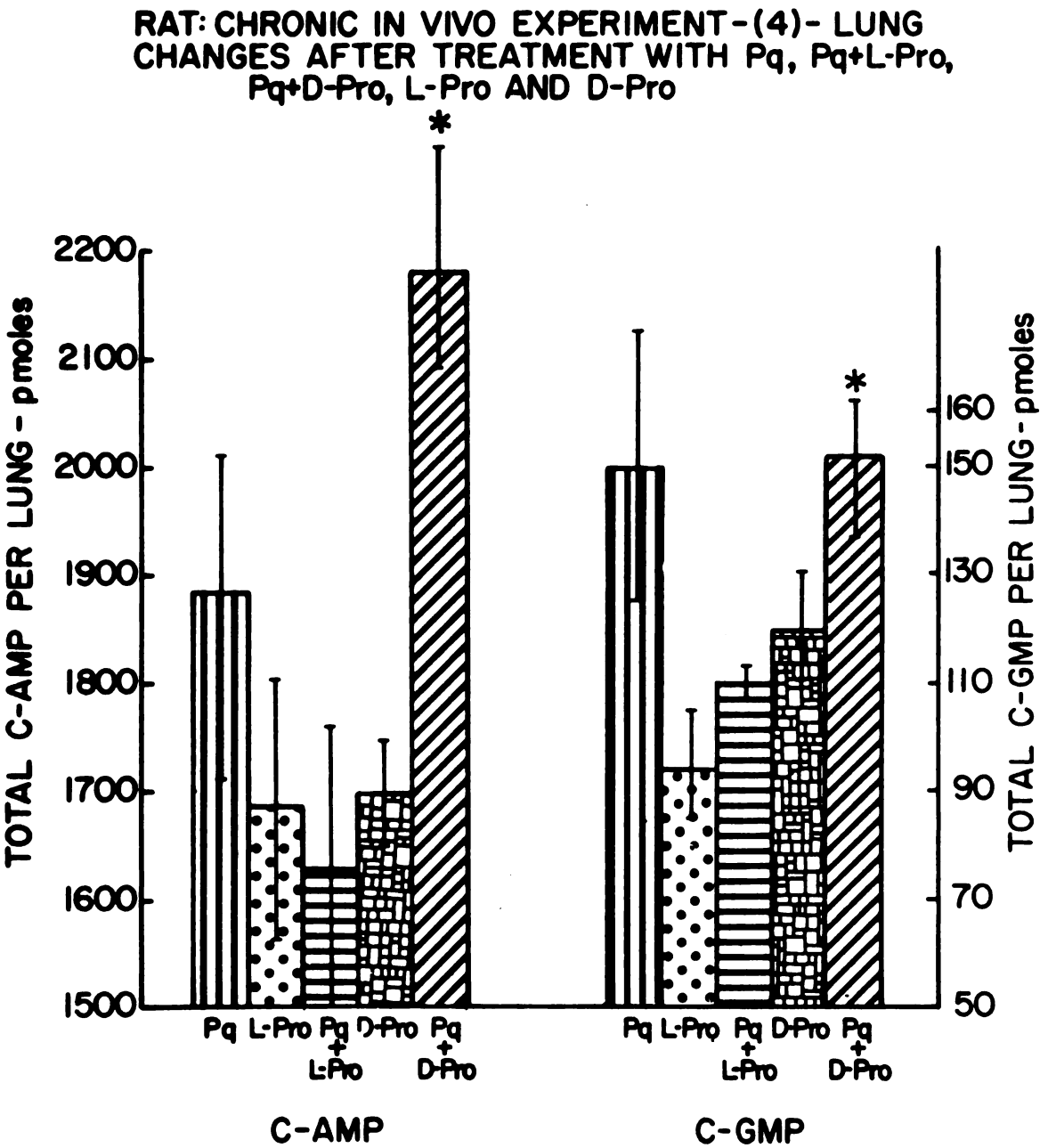


Figure 20. Effect of paraquat, paraquat and l-propranolol, paraquat and d-propranolol, l-propranolol, and d-propranolol on rat lung c-AMP and c-GMP. Rats were treated daily with paraquat (5 mg/kg ip), l-propranolol (5 mg/kg sc 3x), d-propranolol (5 mg/kg sc 3x). On the 10th day all the rats were killed and the lung c-AMP and c-GMP determined. Values represent mean $\pm$ SEM. Asterisk indicates values significantly different from controls ($p < 0.05$).



* Significantly different from controls ($P<0.05$)

Figure 21. Acute effect of paraquat on lung c-AMP and c-GMP. Rats were treated with paraquat (20 mg cation) or saline. Five rats from each group were killed at 30 min, 1.0 hr, 1.5 hr, 2.0 hr, 4.0 hr, 8 hr, 16 hr and 24 hr, and lung c-AMP, c-GMP, paraquat and total proteins determined. Each point represents mean c-AMP, c-GMP or paraquat concentration per mg protein $\pm$ SEM.

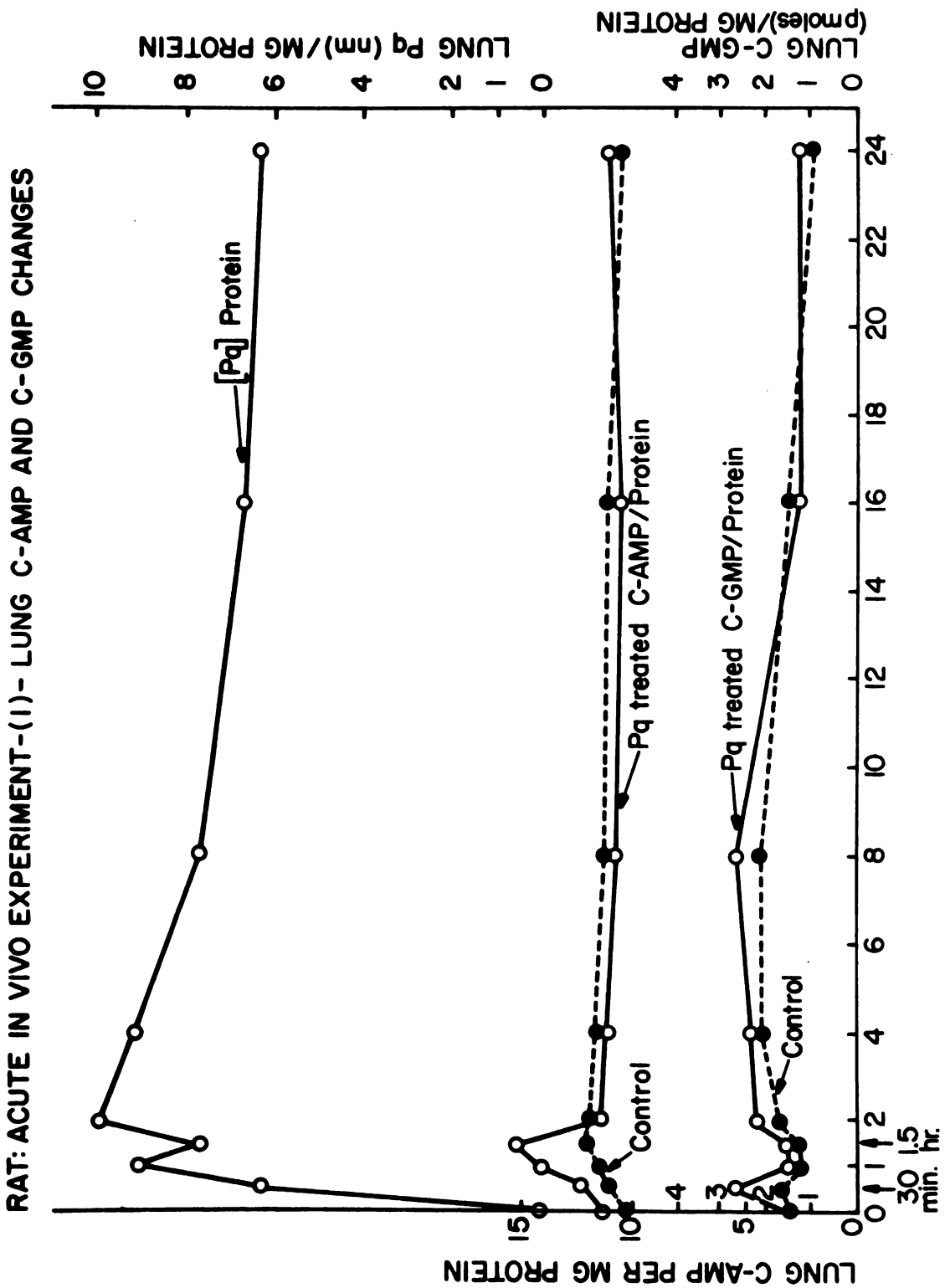

RAT: ACUTE IN VIVO EXPERIMENT-(I)- LUNG C-AMP AND C-GMP CHANGES
LUNG C-AMP PER MG PROTEIN
LUNG C-GMP (pmoles)/MG PROTEIN
LUNG Pq (nm)/MG PROTEIN
[Pq] Protein
Pq treated C-AMP/Protein
Control
Pq treated C-GMP/Protein
Control
0 30 1 1.5 2 4 6 8 10 12 14 16 18 20 22 24
min. hr.

Table 1.--Rat Isolated Lung Perfusion Experiment-Change in Lung Weights.

Experiment	Lung Weight (Gm)	Percent Change In Weight
Paraquat (10)	2.3 ± 0.2	37.3 ± 17.1
Propranolol (10)	2.3 ± 0.1	-3.6 ± 0.3
Paraquat & Propranolol (7)	2.2 ± 0.1	1.4 ± 0.4
Paraquat & Mannitol - 1% (3)	2.8 ± 0.3	6.5 ± 1.8
Histamine (10)	2.9 ± 0.4	2.8 ± 0.4
Perfusate	2.6 ± 0.2	1.0 ± 0.4

The effect of perfusion of different drugs on the change in weight of the lung was studied in the isolated perfused rat lung preparation.

Lungs were weighed before and after perfusion.

Results are expressed as the percent change in lung weight ± standard error of mean.

Values in ( ) refer to number of perfusions.

Table 2.--Summary Rat Chronic In Vivo Experiment 1.

Group	1	2
Treatment	Saline	Pq
No. in Group	25	30
No. Dead	Nil	3
Percent Dead	0	10

Group 2

Day 11 - 2

12 - 1

Total 3

Table 3.--Rat Chronic In Vivo Experiment 1.

Body Weight and Total Lung Protein of Rats Chronically Treated with Paraquat[a]

Day Following Pq Treatment	Body Weight (g)						Total Lung Protein (mg)					
	1	3	6	8	10	12	1	3	6	8	10	12
Control	178.6 ±1.3	190.5 ±1.5	212.0 ±1.6	222.9 ±2.3	243.9 ±1.8	260.4 ±2.5	98.0 ±2.0	101.4 ±4.9	119.4 ±3.0	110.8 ±4.0	118.2 ±3.0	135.8 ±6.4
Treated	179.0 ±1.5	193.0 ±1.9	194.7 ±1.7	206.5 ±2.7	199.9[b] ±4.7	182.8[b] ±5.4	98.0 ±2.0	104.0 ±3.0	130.4 ±5.0	146.4[b] ±5.9	185.8[b] ±14.3	230.3[b] ±13.5

[a]Adult male rats were treated ip with paraquat 5mg/kg daily. Values represent the mean ± mean SEM for 5 rats.

[b]Significantly different from Control ($p < 0.05$).

Table 4.--Summary Rat-Chronic In Vivo Experiment 2.

Group	1	2	3	4	5	6
Treatment	Saline	Pq	Pq + Prop	Prop	Th	Pq + Th
No. in Group	25	30	30	25	25	30
No. Dead	Nil	4	6	Nil	Nil	8
Percent Dead	0	13	20	0	0	27

Group 2 Deaths	Group 3 Deaths	Group 6 Deaths
Day 9 - 1 Died	Day 9 - 1 Died	Day 7 - 1 Died
10 - 2	10 - 2	8 - 1
11 - 1	11 - 0	9 - 2
12 - 0	12 - 3	10 - 2
Total 4	Total 6	11 - 0
		12 - 3
		Total 8

Table 5.--Summary Rat Chronic In Vivo Experiment 3.

Group	1	2	3	4	5
Treatment	Pq	Pq + Prop	Prop	Th	Pq + Th
No. in Group	10	10	10	10	10
No. Dead	Nil	Nil	Nil	Nil	2
Percent Dead	0	0	0	0	20

NB. 3 injections of Propranolol given daily.

Table 6.--Rat Acute In Vivo Experiment 2.

Rat	SA1-5	SB1-5	SC1-5	SD1-5	RA1-5	RB1-5	RC1-5	RD1-5	AA1-5	AB1-5	AC1-5	AD1-5
Mean Total Protein (mg)	124.6 ±2.5	118.6 ±5.2	132.0 ±8.2	125.4 ±5.5	108.2 ±5.3	108.6 ±4.4	115.2 ±3.8	122.4 ±4.1	141.6 ±1.8	142.4 ±1.9	138.0 ±2.9	143.6 ±4.3
Mean Total c-AMP (p mole)	1058.6 ±63.4	1320.0 ±55.8	1588.4 ±63.4	1397.5 ±50.0	1006.4 ±119.0	884.0 ±82.2	1101.6 ±88.4	897.0 ±70.4	545.4 ±46.3	863.8 ±81.6	834.8 ±54.4	821.5 ±35.4
Mean Total c-GMP (p mole)	335.4 ±14.8	361.6 ±17.3	423.8 ±8.4	424.4 ±7.4	457.8 ±17.7	440.2 ±8.4	453.0 ±14.6	433.2 ±6.8	409.0 ±15.0	413.2 ±9.4	390.0 ±12.6	391.8 ±17.0
Mean c-AMP/ Protein	8.46 ±1.00	10.05 ±0.84	12.13 ±1.13	11.48 ±1.00	9.44 ±1.30	8.28 ±0.60	9.52 ±0.54	8.65 ±0.30	3.86* ±0.36	6.90* ±0.90	5.80* ±0.35	5.68* ±0.35
Mean c-GMP/ Protein	2.70 ±0.17	3.06 ±0.30	3.26 ±0.20	3.41 ±0.20	4.21* ±0.40	4.08* ±0.18	3.90 ±0.20	3.60 ±0.80	2.89 ±0.12	2.90 ±0.06	2.83 ±0.10	2.73* ±0.10

Values represent the Mean ± SEM for 5 rats.

*Significantly different from control ($p < 0.05$).

0 time - SA1-5: RA1-5: AA1-5
30 min - SB1-5: RB1-5: AB1-5
1 hr - SC1-5: RC1-5: AC1-5
2 hr - SD1-5: RD1-5: AD1-5

Table 7.--The Effect of Paraquat on Cyclic Nucleotides in Thymus, Liver, Spleen, Kidney, and Lung.

		Mean c-AMP (p mole) / Protein (mg)	Mean c-GMP (p mole) / Protein (mg)
Thymus	control	10.78 ± 0.74	1.83 ± 0.04
	treated	11.30 ± 0.71	1.88 ± 0.23
Kidney	control	5.91 ± 0.35	1.15 ± 0.10
	treated	6.28 ± 0.21	1.31 ± 0.90
Liver	control	1.98 ± 0.20	0.33 ± 0.03
	treated	2.49 ± 0.17	0.44 ± 0.16
Spleen	control	6.71 ± 0.32	1.15 ± 0.05
	treated	6.30 ± 0.37	1.45 ± 0.11
Lung	control	8.46 ± 1.00	0.38 ± 0.16
	treated	20.38 ± 1.22*	2.23 ± 0.69*

Values represent Mean ± SEM for 3 rats.

*Values significantly different from control ($p < 0.05$).

Male Sprague-Dawley rats were treated with paraquat (5mg/kg) ip daily for eight days. Control rats received saline ip daily for the same period. Rats were anesthetized and the thymus, kidney, liver, spleen, and lung were removed and quickly frozen in liquid nitrogen. These specimens were homogenized in 10% TCA and assayed for c-AMP, c-GMP, and total proteins.

Table 8.--The Rf Values of Prostaglandin PGE_2 and Its Three Metabolites in TLC System.

Compound	Rf Value
PGE_2	0.186 ± 0.011
15-Keto PGE_2	0.567 ± 0.026
13,14-dihydro-15-keto PGE_2	0.747 ± 0.025
13,14-dihydro PGE_2	0.260 ± 0.016

Values represent mean ± SEM for 5 determinations.

Twenty µl of each compound was applied to the silica gel G thin-plate chromatogram (Analtech, Newark, Del.). The plates were developed in chloroform:tetrahydrofuran:acetic acid (100:10:5 by vol) for 80 min. The solvent front travelled 15 cm from the origin. The plates were sprayed with 5% phosmolybdic acid and dried at 120°C for 15 min.

Table 9.--Effect of Paraquat on Prostaglandin E_2 Metabolism by the Rat Isolated Lung (Using TLC).

	Venous Effluent			Lung Homogenate		
Compound	PGE_2	15-Keto-PGE_2	13,14 dihydro 15-keto-PGE_2	PGE_2	15-Keto-PGE_2	13,14 dihydro 15-Keto-PGE_2
Control	15.40±1.22	57.67±1.23	8.01±1.18	24.38±0.78	44.78±3.40	7.13±0.78
Treated	28.52*±4.16	45.34*±3.55	8.42±1.20	16.30*±2.08	48.36±4.16	9.20±1.74

Values represent Mean ± SEM for 5 samples.

*Values significantly different from control ($p < 0.05$).

Table 10.--Effect of Paraquat on the Amount of Prostaglandin E_2 Metabolized by the Rat Isolated Lung.

	PGE_2 Metabolized (%)	15-keto-PGE_2 Formed (%)	13,14-dihydro-15 Keto PGE_2 Formed (%)
Control	72.30 ± 1.22	56.30 ± 1.23	4.59 ± 1.19
Treated	59.18* ± 4.16	44.57 ± 3.55	5.10 ± 1.20

Values represent Mean ± SEM.

*Values significantly different from control ($p < 0.05$).

Data drived from Table 9 by substracting correspdong values obtained from perfusion experiment without rat lung.

DISCUSSION

The isolated perfused lung is a useful model for studying a chemical compound like paraquat which has the lung as its main target organ. On perfusing the rat isolated lung with bovine serum albumin-Kreb's Ringer bicarbonate and measuring the glucose content of the perfusate over the period of study, it was observed that the glucose concentration steadily decreased with time, a change that was not observed when the lung was not included (Figure 4). This has been used as a criterion of viability of the lung in conjunction with other gross anatomical criteria.

The isolated rat lung perfused with paraquat, propranolol, histamine, paraquat and propranolol, or paraquat and mannitol, showed different degrees of edema, as measured by the percentage change in lung weight. Paraquat produced a rapid and marked edema during the 90 minute period of perfusion and in the presence of propranolol and mannitol, the edema produced on perfusing the lung with paraquat was significantly reduced (Figure 3).

This observation suggested a protection of the lung from the toxic effect of paraquat although this was not borne out by the rat in vivo studies.

Although the isolated lung is a useful model for studying the effect of a chemical agent with a specific effect on the lung, in the

final analysis the only means of establishing an effect unequivocally is by its effect in the intact animal. It is possible to explain the effect of propranolol in reducing the edema observed on perfusing paraquat, on the basis of the fact that both paraquat and propranolol are basic lipophilic amines with similar physico-chemical properties and known to be extensively accumulated by the lung and to competitively inhibit the uptake of each other. Other amines including biogenic amines may behave in a similar manner. The rate of accumulation of paraquat by rat lung from plasma *in vivo*, following oral dosing was about one seventh of that predicted from *in vitro* (Rose *et al.*, 1976). This observation led to the suggestion that inhibitors of paraquat accumulation may be present in the circulation. A number of endogenous amines including norepinephrine, 5-hydroxytryptamine, and histamine, have been reported to reduce concentrations of paraquat accumulation into lung slices, as have several other drugs including imipramine, d-propranolol, betazole, diquat, and burinamide (Lock *et al.*, 1976). No precise structural requirement has emerged for compounds which inhibit the accumulation of paraquat, although the substitution of a carboxyl group on the α-carbon carrying the amino group to an amino acid (e.g., tyramine to tyrosine, histamine to histidine), abolishes the ability of the amine to inhibit paraquat (Lock *et al.*, 1976).

Lock *et al.* (1976), have suggested two types of inhibition, namely the inhibition produced by norepinephrine, imipramine, diquat and lysine which is linear with time and may be explained by simple competition between paraquat and inhibitor at the uptake site, and a non-linear inhibition produced by betazole and histamine.

An understanding of the mechanism of inhibition of paraquat uptake is important in the search for agents that reduce the uptake of paraquat into the human lung. Compounds which will be of therapeutic value will be those which can be given in quantities sufficient to inhibit paraquat accumulation into the lung, until such time that the paraquat is excreted from the body or removed by other therapeutic measures.

The lung accumulates paraquat very much more effectively than any other organ examined. This selectivity must be a primary factor in the development of lung damage and partially explains why this organ is the most severely effected.

The main lung cell types that accumulate paraquat are the alveolar epithelial type I and II cells (Ilett et al., 1974).

Mannitol, a polyhydric alcohol used mainly as an osmotic diuretic is an effective scavenger of hydruxyl radicals as it has a protective effect against hydroxyl (OH$^{\cdot}$) depolymerizing species generated secondarily by a reaction between the enzymatically generated superoxide radical from peroxidation pathway and hydrogen peroxide (The Haber and Weiss reaction) in hyaluronidase deploymerization (McCord, 1974). On the basis of the hypothesis that lipid peroxidation is the underlying mechanism in paraquat toxicity, mannitol was tried out and it was interesting to observe that it significantly reduced the edema from paraquat perfusion.

Perfusing the isolated rat lung with isoproterenol or paraquat resulted in an increase in the lung cyclic AMP and perfusing paraquat and propranolol resulted in significantly less cyclic AMP in the lung (Figure 6). This finding was also observed in the in vivo experiments.

Kuo and Kuo (1973), studied the effects of adrenergic and cholinergic agents on the levels of cyclic AMP and cyclic GMP in slices of rat lung. They reported that isoproterenol significantly increased pulmonary cyclic AMP levels (about three fold) and this increase was abolished by propranolol but not by phenoxybenzamine. Stoner et al. (1973), reported that pulmonary cyclic AMP was markedly increased by exposure to isoproterenol, epinephrine, or prostaglandin E_1.

The rat in vivo experiments carried out in this project involved acute and chronic exposure to paraquat. Weight loss and lethality were the most useful indices of paraquat toxicity in these experiments. In all the chronic experimemtns, weight loss was observed in rats which received paraquat by itself or together with propranolol or theophylline. There was in the chronically treated rats a slower weight gain compared to control rats but after about the sixth day of chronic treatment progressive weight loss was observed and this was more marked in rats which received paraquat and theophylline than those which received paraquat alone or paraquat and propranolol (Figure 11). Mortality in the paraquat and theophylline treated rats was higher but not significant at the 5% level compared to the controls (Figure 6). Weight loss associated with paraquat poisoning is due to failure of many paraquat treated animals to accept food.

Lung total proteins in paraquat treated rats showed a steady and progressive increase in all the chronic experiments (Figure 8). This increase in lung protein is due to increase in protein synthesis and Van Osten and Gibson (1975), correlated it with the proliferation of endoplasmic reticulum and the enlargement of "ribosomal" granules. They further suggested that the synthesis of cellular protein and

nucleic acid components in paraquat poisoned animals may be secondary to paraquat initiated free radical toxicity rather than the direct result of paraquat action on cellular control mechanisms.

The exact mechanism of the lung protein increase observed on chronic paraquat treatment is not clear. This observed increase in lung protein concentration makes it unacceptable to express specific values such as cyclic AMP in terms of mg. of protein. Thus, in the chronic experiments in this project, it seemed that to express data per total lung was the most satisfactory approach. Figures arrived at this way will not be affected secondarily by changes in chemical composition of the lung and therefore they allowed one to compare the entire biochemical capacity of the damaged lung with the values found in the appropriate controls.

Maling et al. (1975), reported what appeared to be an encouraging result from the use of the β-adrenergic blocking drug, propranolol, in rats. Since ingestion of paraquat in humans produced respiratory distress, it was considered reasonable to treat paraquat poisoned rats with the β-adrenergic agonist, l-isoproterenol to dilate the bronchi and improve gaseous exchange. However, it was found that treatment with l-isoproterenol (Maling et al., 1975) or salbutamol (Fletcher, 1973), increased the lethal effect of paraquat. Theophylline, a xanthine derivative, also produced a potentiating effect with paraquat. Maling et al. (1975), reported further that pretreatment with dl-propranolol or l-propranolol was relatively ineffective. These findings suggested the β-adrenergic receptor may be involved in the toxicity of paraquat since it is known that the β-adrenergic receptor blocking activity is associated mainly with the L-isomer (Barret, 1969). Other

β-adrenergic blocking agents including bunolol, sotalol, pronethalol, and KO592 also reduced the mortality from paraquat although bunolol and propranolol proved more effective than the others.

In the light of the above findings, a part of this project was designed to find out whether cyclic AMP, which is involved in the mediation of most of the metabolic effects of the β-adrenergic system, and the other naturally occurring cyclic nucleotide, cyclic GMP, have a role in paraquat toxicity. It was thus interesting to observe increases in pulmonary cyclic AMP and cyclic GMP on treatment with paraquat.

The rat chronic in vivo experiment 1 showed that paraquat significantly increased intracellular cyclic AMP (by two fold), (Figure 9) and cyclic GMP (by ten fold), (Figure 10). This observation was found in subsequent in vivo experiments and theophylline was observed to potentiate the increases in these cyclic nucleotides. Thus, rats treated with paraquat and theophylline showed a rapid and greater weight loss compared to controls (Figure 11), and significantly higher pulmonary paraquat concentrations (Figure 16), although the lung paraquat concentrations were not consistently statistically different from the other groups in some of the experiments.

The mortality in the paraquat and theophylline treated rats (27%), was higher but not significantly different from rats treated with paraquat only (13%), or with paraquat and propranolol (20%), (Table 6). Rats treated with paraquat and theophylline had significantly less pulmonary theophylline compared to rats which only received theophylline (Figure 17). The protection afforded by propranolol in reducing paraquat induced pulmonary edema in the isolated lung

experiment was not reproduced in the *in vivo* experiments. This observation may be due to a difference in rate of uptake of propranolol *in vivo* compared with that *in vitro*. This may be the result of propranolol uptake inhibitory substances in plasma, binding of propranolol by components of plasma or increased metabolism of propranolol to less active metabolites thus reducing the concentration of free propranolol. Differences in the behavior of lung *in vivo* compared with the isolated perfused lung cannot be ignored. The pulmonary paraquat concentration was not significantly different from rats treated with only paraquat but the propranolol concentration was significantly higher (by half as much) in rats which received paraquat and propranolol compared to rats which received only propranolol (Figure 16). Gardiner and Shanker (1976), reported that the permeability of the lung is markedly increased in the presence of paraquat induced damage and pointed out that relatively lipid insoluble compounds primarily absorbed by diffusion through aqueous membrane pores showing marked increase in absorption rate by paraquat damaged lungs, is consistent with the idea of an increased porosity for the absorbing membrane. This explanation applies to propranolol which is a relatively lipid insoluble compound.

In the case of theophylline, a lipid soluble drug, increased membrane porosity would be expected to have a lesser effect on the absorption rate, since although diffusing through pores, the drug is absorbed predominately by crossing lipid regions of the pulmonary membrane (Enna and Shanker, 1972). Other factors which could contribute to changes in membrane permeability of paraquat damaged lungs to drugs include reduced pulmonary surfactant activity (Manktelow, 1967),

and a change in pulmonary blood flow. The presence of edema fluid itself could slow absorption rates owing to the decreased absorbing surface/volume ratio.

In the chronic in vivo experiments other organs including the liver, spleen, thymus, and kidney, which are known to be affected by paraquat were assayed for cylic AMP and cyclic GMP and compared to controls. It was found that the cyclic nucleotide content of these organs was not significantly different from controls, while it was significant in the lungs (Table 7). This finding suggested the pulmonary cyclic nucleotide changes observed were probably related to the peculiar toxic effect of paraquat on the target organ, the lung.

Acute in vivo experiment II was designed to determine whether increased endogenous catecholamines were responsible for the increased cyclic nucleotides observed in the lung.

Rats pretreated with reserpine to deplete catecholamine stores, then given paraquat had lung cyclic AMP values not significantly different from controls (Table 6). This observation suggested that endogenous catecholamine was not a factor in the cyclic AMP elevation in the lung discussed above. On the other hand, rats pretreated with atropine followed by paraquat had pulmonary cyclic AMP values significantly lower compared to controls (Table 6).

Kuo and Kuo (1973), reported that increases in cyclic GMP levels in the lung tissue are closely regulated by muscarinic cholinergic receptor activation, elevated by acetycholine and abolished by atropine. Acetylcholine also increased the cyclic AMP content. Thus, in the

present study it was observed that atropine abolished an increase in cyclic GMP after paraquat treatment and cyclic AMP level was significantly reduced compared to controls (Table 6).

It is rather difficult to assign specific sites or functions to the changes in cyclic nucleotide concentrations in a tissue as heterogenous as the lung. Stoner et al. (1973), suggested that increased cyclic GMP concentrations produced by acetycholine occurred in cells innervated by the parasympathetic nervous system, that is, cyclic GMP serves as a "second messenger" for acetylcholine. The position with cyclic AMP is much more complicated since the lung is a site for synthesis or storage of a number of agents that can increase cyclic AMP concentration, for example, norepinephrine, prostaglandins, and histamine. Alternatively, the accumulation of cyclic GMP might itself increase cyclic AMP concentration as has been shown to occur in fat cells, kidney slices, and avian erythrocytes (Murad et al., 1970), presumably by inhibiting cyclic AMP phosphodiesterase (Ferrendelli et al., 1970). The Acute in vivo Experiment I showed an increase in cyclic GMP with a peak at 30 minutes followed by a cyclic AMP peak at 1.5 hours after paraquat treatment.

Recently there has been increased awareness of the role played by the lung in regulating the systemic arterial blood levels of important vasoactive hormones including prostaglandins, peptides, and biogenic amines. Such a regulation may be achieved by uptake and metabolism of substances reaching the lung via the pulmonary artery, for example, prostaglandins of the E and F series, serotonin and bradykinin, or by synthesis within the lung e.g., angiotensin.

The isolated lung from paraquat treated rats perfused with ^{3}H-PGE_2 showed significant inhibition of PGE_2 metabolism. This may be due either to an impairment of the uptake mechanism of PGE_2 to the site of metabolism or to interference with the enzymes involved in prostaglandin metabolism. Bito and Baroody (1975) suggested that the capacity of the lung to metabolize PGs required the transmembrane transport as an initial step. Thus, any interference with that mechanism would reduce the amount of PG metabolized. There is also evidence that the capacity of the lung to metabolize PGs can be modified by various conditions and changes which affect the enzymes involved in the metabolic processes. Parkes and Eling (1975) reported that the activity of the enzyme, prostaglandin dehydrogenase (PG-DH) in the lung is reduced on exposure to 100% oxygen or endotoxin shock.

Interference with the pulmonary mechanism for inactivating endogenous vasoactive hormones such as the PGs by drugs, toxic chemicals, atmospheric pollutants or disease may be more important than hitherto appreciated. In paraquat poisoning, interference with excretion as a result of circulating abnormal levels of vasoconstrictor hormones would accentuate toxic effects particularly on the target organ, the lung.

SUMMARY

Paraquat, is a bipyridylium compounds used as a broadspectrum herbicide, which is very toxic to the mammalian species. The lung is the main organ affected by paraquat although other organs such as the kidney, liver, spleen and thymus may be affected to a lesser extent.

Perfusing the rat isolated lung with paraquat resulted in a rapid and marked edema which was significantly reduced by propranolol and mannitol.

This protective effect was not observed in the in vivo experiments.

Paraquat stimulated increased levels of the cyclic nucleotides, cyclic AMP and cyclic GMP, both in the rat isolated lung and in vivo. Theophylline potentiated the increase in cyclic nucleotides in the lung. The cyclic AMP changes observed in the lung were not affected by pretreatment with reserpine but atropine pretreatment significantly reduced pulmonary cyclic AMP.

The isolated lung from the paraquat treated rats perfused with ^{3}M-PGE_2 showed significant inhibition of PGE_2 metabolism, probable due to impairment of the uptake mechanism for PGE_2 to the site of metabolism.

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