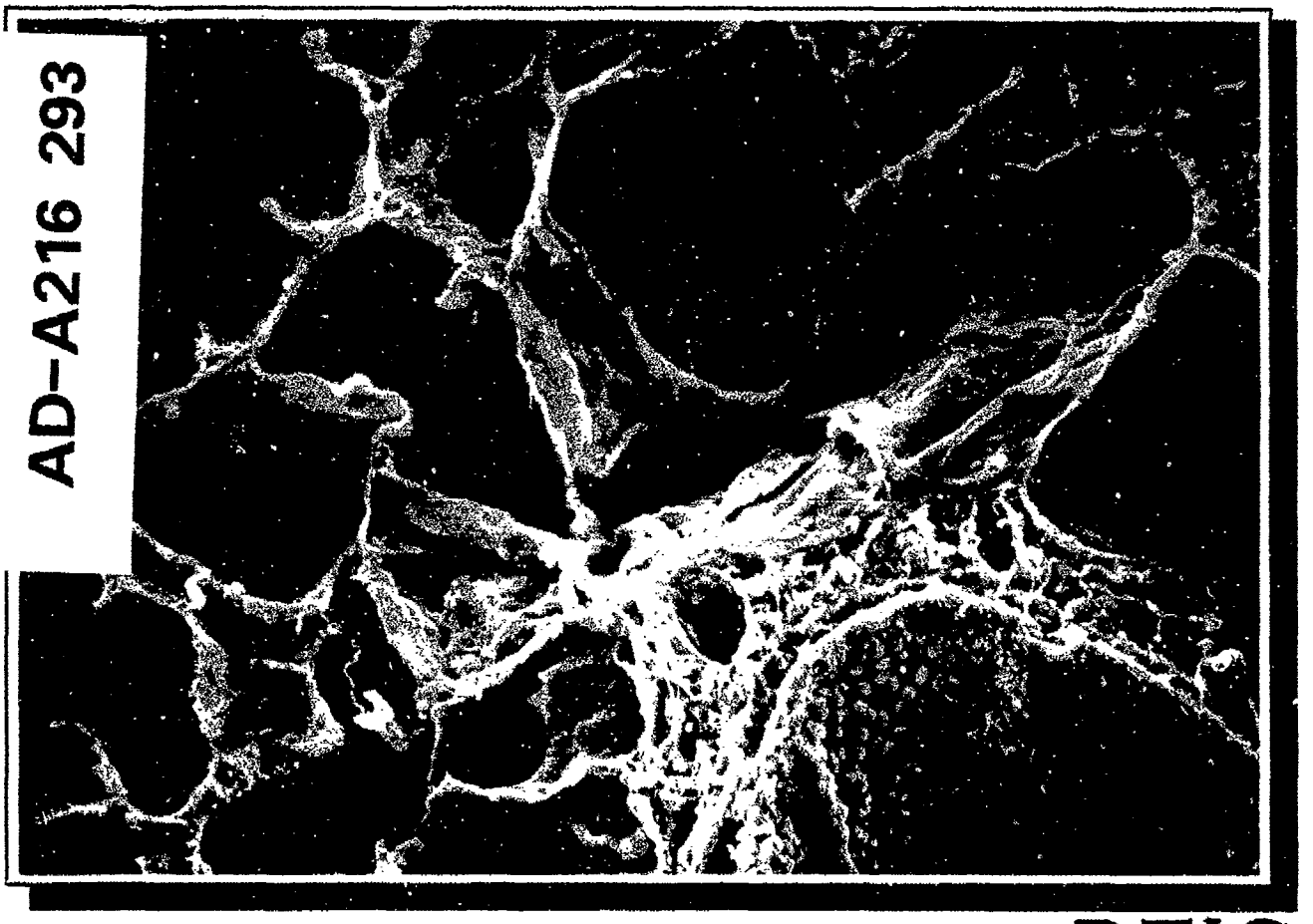
Proceedings
of the
Workshop on Acute Lung Injury and Pulmonary Edema

4-5 May 1989
Aberdeen Proving Ground, Maryland



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Cover photograph: *Normal lung showing bronchiole and surrounding alveoli (magnification 310X). Provided by Dr. John P. Petrali.*

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The workshop on Acute Lung Injury and Pulmonary Edema was held at Edgewood Arsenal on 4-5 May 1989. The objective of the workshop was to review the current state of knowledge concerning organohalide-induced pulmonary intoxication. This document constitutes the proceedings of that workshop.

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PROCEEDINGS

of the

WORKSHOP ON ACUTE LUNG INJURY

AND PULMONARY EDEMA

Edgewood Arsenal, Aberdeen Proving Ground, Maryland

4-5 May 1989

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PREFACE

The role of the lungs as a portal of entry for many chemical warfare agents has been recognized for some time. During the past decade, however, even greater emphasis has been placed on the importance of the respiratory tract in medical chemical defense.

An essential mission of the USAMRICD is to develop medical countermeasures against chemically induced lung injury. Accordingly, this workshop was organized to provide a forum in which experts in this field could share their information and insights into a particular class of chemicals--the organohalides. Some chemical agents, such as organophosphates and cyanide, enter the body through the respiratory system, but their direct toxic effects are expressed at other vital locations throughout the body. The primary toxicity of the organohalides appears to be at the level of the lung itself. Included in this class of chemicals are phosgene, historically one of the oldest chemical warfare agents (one which is still often used as a standard to which other respiratory agents are compared), and newly recognized industrial byproducts with toxicities similar to that of phosgene.

The participants in this workshop include investigators from the USAMRICD who have been conducting preliminary research on the pathophysiologic effects of organohalides and investigators from academia who have research experience in the field of lung injury.

We acknowledge the valuable contributions of the workshop participants. We are indebted also to Dr. Donna Bareis and Dr. Gary Kiebzak of SAIC for their outstanding support in the organization of this workshop and in the preparation of the proceedings.

DAVID H. MOORE
MAJ, VC
CHIEF, PATHOPHYSIOLOGY DIVISION

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OPENING REMARKS
(Abridged)

COL Michael A. Dunn

I would like to welcome those of you who are not assigned here and have not visited our Institute before. I consider this audience one of the most important working groups of outside investigators that we have assembled this year or are likely to assemble for some time to come. I hope that my opening comments will indicate why I feel this way.

MAJ Moore will give you an overview of the Institute's structure, function, and mission. By way of introduction, I will first tell you that our mission is to conduct research leading to medical countermeasures against recognized chemical warfare agents and against compounds that we have strong reason to believe may be under development by enemies as chemical warfare agents. It is well known that this is the mission of the Institute. I would like to emphasize, especially to our first-time visitors, that we do not conduct research here to support U.S. weapons development. This is a function of other organizations in the Army. As you all know, the United States has had a retaliatory chemical weapons capability for the last 72 years, but none of the content of this workshop, and none of what we do, is concerned with the support of weapons development. Our mission is the development of medical countermeasures, pretreatments, antidotes, and casualty treatment strategies.

About 3 years ago, the Institute became involved in research on the general topic of the pulmonary toxicity of a class of compounds called the organofluorines [organohalides]. Perfluoroisobutylene (PFIB) is the compound of that class that we first started to work with. We initiated a small program of classified research within the Institute. We had four of the finest investigators in the Institute working on this program: two physiologists, LTC Jim Jaeger and MAJ Dave Moore; a pathologist, LTC Nancy Jaax; and a clinician-bioengineer, MAJ Ken Phillips. That small group of people have made fine progress in defining the nature of the organofluorine-induced lung lesions at the light- and electron-microscopic levels, the physiological stressors that might contribute to a lesion, and some of the physiological and clinical counterparts of the lesions or series of lesions as they evolve. Gradually, we have expanded our efforts in this area. We are confident that, given open interaction with the scientific community, we can further our knowledge of fundamental molecular mechanisms and identify some new approaches to therapy.

When we reached the stage at which we thought it necessary to bring in investigators and expertise from outside the Institute, it was no longer appropriate to treat this work as classified. After this decision was reached, we invited investigators from other government laboratories and from academia to this unclassified meeting with the aim of expanding the capability of the people currently working on the pulmonary toxicity of organofluorines. As a consequence of this meeting, I would like to see progress toward answering the questions: What is the molecular mechanism of the organofluorines? What is going on at the subcellular and molecular levels? What are the interventions that one could contemplate both in the latent period--the presymptomatic period that you are going to hear about--and, if possible, after symptoms have developed? Is there a capability, for example, during the symptom-free latent period to implement dosimetry or other clinical procedures that would separate those persons at high risk for development of major lesions from those who are essentially at no risk and could continue to perform their duties?

In the event that you desire information in addition to what I have just given you--for example, about the operational characteristics of the organofluorines--I encourage you to talk to me or to MAJ Moore. I think we have access to some minds that could help us make substantial progress in this area. I have great expectations and high hopes for progress resulting from this meeting. Thanks very much for being here.

OPENING REMARKS
(Abridged)

MAJ David H. Moore

I. U.S. Army Medical Research Institute of Chemical Defense (USAMRICD): Overview of Organization, Goals, and Research Activities

I would like to welcome all of you to this workshop. For the sake of those who are unfamiliar with our Institute--where we fit into the U.S. Army and our place in the Medical Research and Development Command--I will take a few minutes to describe this organization.

The Institute of Chemical Defense at Edgewood Arsenal is one of a number of laboratories under the Headquarters of the U.S. Army Medical Research and Development Command in Frederick, Maryland. Among sister laboratories are the Letterman Army Institute of Research (LAIR), the Walter Reed Army Institute of Research (WRAIR), and the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID).

The mission of USAMRICD can be broken down into three primary goals. The first is to maintain a technology base which comprises capabilities that meet present requirements, and which allows us to respond to potential threats. The second is to provide individual-level prevention/protection, which includes pretreatment compounds and antidotes. Examples are oxime reactivators, cholinolytics, and anticonvulsants for organophosphates. Our third goal is to provide medical management techniques for chemical casualties. We conduct a course in the medical management of chemical casualties here at the Institute for DoD medical personnel. USAMRICD is the lead laboratory in medical chemical defense for the entire Department of Defense, not just for the Army.

To accomplish our mission, we have a staff of approximately 285 at the Institute, a large percentage of whom have degrees in biomedical science. A wide range of disciplines is represented here--for example, library sciences and economics. About three-fourths of the personnel are civilian; about one-fourth are active-duty military personnel. We feel that we have a well-rounded, self-contained research institute.

The Institute is organized into divisions under the Office of the Commander. We have an Administrative Division, a Veterinary and Laboratory Resources Division, and a Research Operations Division. The research portion of the Institute is then divided into three primary research divisions: Pathophysiology, Drug Assessment, and Pharmacology. Along with each research division is a research Task Area. The division chiefs are the task area managers. I am the chief of the Pathophysiology Division; it's within this division that much of the organofluorine research up until now has been conducted. We are now expanding that research and are getting input and active participation from some of the Institute's other research divisions.

What sets our mission in the Pathophysiology Division apart from the work of other research divisions is that we are interested primarily in mechanisms of action--not in the actual testing or screening of pretreatment and therapeutic compounds. Rather, we look at mechanisms of action for both the chemical agents themselves and for the proposed treatments and therapeutic agents. Another mission of our division is the management of a task area, the Pathophysiology Task Area, which represents a large number of extramural research contracts with academic institutions--some of which are represented here today, some of which are in foreign countries; we also contract with private laboratories and with other governmental agencies. In addition, we maintain a technological base within-house; we currently have approximately 35 active in-house research protocols within the Pathophysiology Division.

Pathophysiology comprises three research branches. Within the Physiology Branch, we have efforts in each of the major threat areas: nerve agents, vesicating agents, respiratory agents, and blood agents. Primary emphasis over the last few years has been on the respiratory agents.

The Comparative Pathology Branch has a dual role. Approximately half of their effort goes into supporting research throughout the Institute; the other half goes into independent research projects.

In the past, the Neurotoxicology Branch has placed primary emphasis on organophosphates. Now, the branch is subdivided into an integrated neural systems team and a membrane biophysics team. The integrated neural systems team looks primarily at anticonvulsants and agent-induced convulsant activity, while the membrane biophysics team currently focuses much of its efforts on the biotoxins and their effects on channel activity.

There, in a nutshell, is an overview of the U.S. Army Medical Research Institute of Chemical Defense--the organization for which you, as contractors, are producing research.

II. Chemicals that Produce Pulmonary Edema

A number of chemicals, when inhaled, result in pulmonary edema. The condition usually manifests itself after a symptom-free period, and it varies with duration of exposure and with the dose of the inhaled toxicant. Among the toxicants are the oxides of nitrogen and several of the organohalide compounds, some of which we have been studying here at USAMRICD, and on which we will focus during most of this workshop.

Perfluoroisobutylene (PFIB) is often encountered in industry as a pyrolysis product of tetrachlorylethylene polymers such as teflon (Figure 1).

The second major organohalide of interest is bis(trifluoro-methyl)disulfide (TFD) (Figure 2). TFD is often encountered in industry and agriculture because it is used in the preparation of stable polyfluorinated fluids, such as refrigerants and coolants, and as a fungicide and fumigant.

Carbonyl chloride, more popularly known as "phosgene," (Figure 3) is used by industry in the synthesis of various chemicals, dyes, and pesticides, and in metallurgy for separating ores. The United States produces over a million pounds of phosgene per year. Of these three compounds, only phosgene is a recognized chemical warfare agent, used initially by the Germans in World War I. One would assume that the mechanism of toxicity of an agent as old as phosgene would be well-known by now, but this is not the case.

Our aim, at the Institute of Chemical Defense, is to compare and contrast these three semirelated compounds in order to define the mechanisms involved in the pathogenesis of the acute lung injury induced by their inhalation. The limited data we have suggest that there are similarities in their mechanisms as well as subtle differences, just as there are differences in pulmonary features between shocked lung, neonatal respiratory distress syndrome, and endotoxemia.

This morning's presentations will cover, in chronological order, the research projects that have been conducted in-house over the last several years.

perfluoroisobutylene (PFIB)

Figure 1

bis-trifluoromethyldisulfide (TFD)

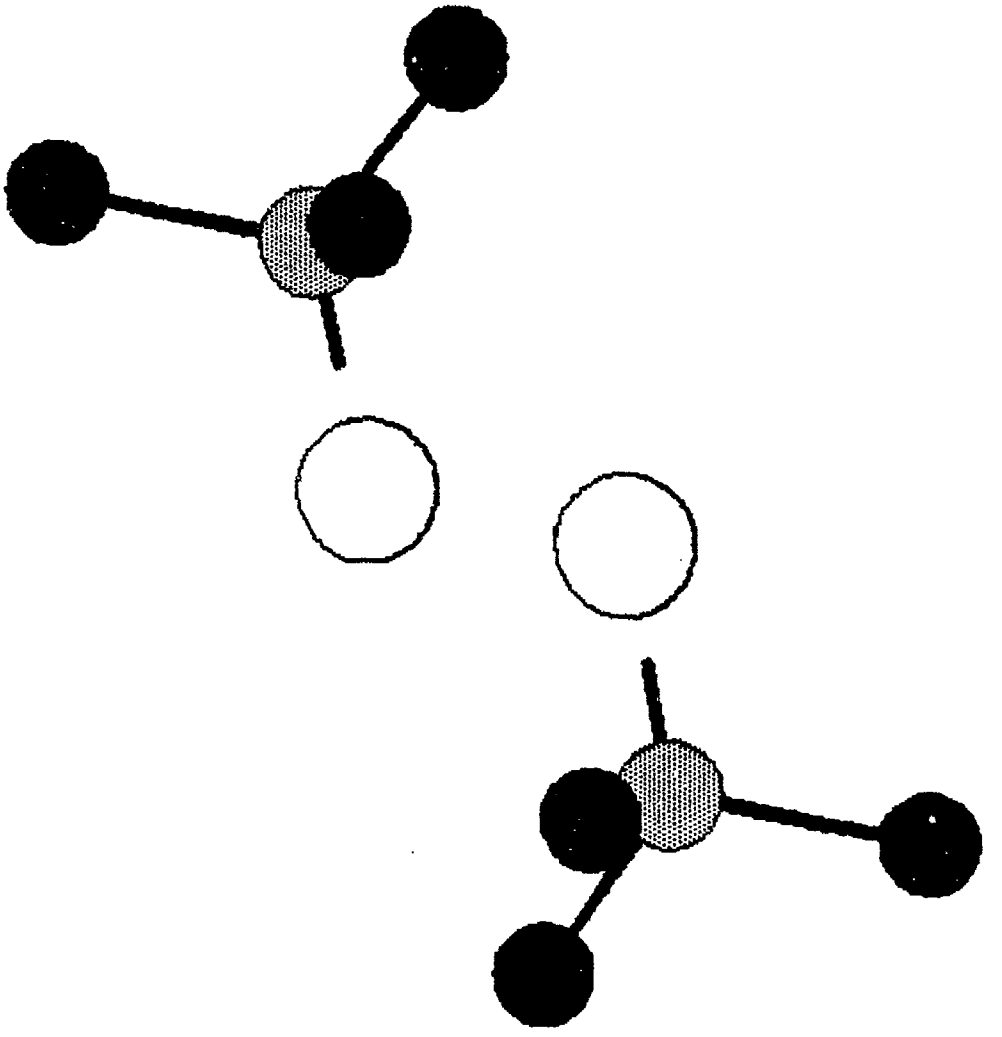

Figure 2

carbonyl chloride (phosgene)

Figure 3

Presentations

EFFECTS OF EXERCISE ON PULMONARY INJURY INDUCED BY TWO ORGANOHALIDES IN RATS

MAJ David H. Moore

These studies were performed to determine whether exercise potentiates the effects of the two organohalides PFIB and TFD. This is of military interest since these compounds are potential health threats, and it is likely that soldiers exposed to these agents will participate afterward in strenuous physical activity.

Rats were adapted to rapid treadmill running and randomly divided into four experimental groups: (1) exposure to PFIB (100 mg/m^3 for 10 minutes) or TFD (200 mg/m^3 for 10 minutes) followed in 15 minutes by running to exhaustion; (2) exposed then rested; (3) sham-exposed followed by running, and; (4) sham-exposed then rested. Doses represented the approximate LCt_{50}. Rats were sacrificed 24 hours after exposure. Lungs were collected and wet and dry weights were recorded in order to assess the development of edema. Samples of the right apical lobes were collected and submitted for histopathological examination.

Exposure to the organohalides did not result in significant decrements in running endurance (i.e., running to exhaustion). However, exposure to both compounds resulted in the development of pulmonary edema and associated lung pathology. This effect was potentiated by exercise. Thus, both exposure and exercise effects were found. Finally, TFD was found to be extremely toxic; 11 of 12 exercised rats died within 24 hours after exposure.

EFFECTS OF EXERCISE ON PULMONARY INJURY INDUCED BY TWO ORGANOHALIDES IN RATS

MAJ David H. Moore

METHODS AND MATERIALS

Experimental Animal:

Age-matched male Sprague-Dawley rats (250-350 g) were obtained from Walter Reed Army Institute of Research and housed in the central animal facility for at least five days prior to delivery to the laboratory. The animals were housed in pairs in animal quarters where the light/dark cycle (12 hrs/12 hrs) and temperature (24°C) were automatically controlled. The animals had access to food (Formulab Chow #5008), Ralston Purina Co., St. Louis, MO) and water ad libitum.

Experimental Design

Serial Sacrifice:

Forty-eight rats received whole body exposure to a concentration of 110 mg/m^3 of PFIB for 10 minutes. Groups of three animals each were sacrificed at 5 minutes, 30 minutes, 90 minutes, 4 hours, 12 hours, 24 hours and 72 hours post-exposure. Eleven animals were sacrificed at 7 days. Twelve animals were sacrificed at 14 days. Five unexposed animals were sacrificed as pathologic controls without sham exposure.

Effect of Exercise:

From a group of 48 rats, animals were randomly assigned to one of four experimental groups. Groups one and two were exposed to PFIB 1000 mg-min/m^3. Groups three and four received sham whole body exposures. Groups one and three were exercised to exhaustion 30 minutes post-exposure, while groups two and four remained sedentary. To acclimate the animals to running on the treadmill (Omnitech, Speed-a-matic, Emerson Electric Co., Columbus, Ohio), all animals, regardless of group, were exercised at 35 m/min and 0% grade for 10 minutes a day, 5 days a week, 3 weeks prior to exposure (2). Rats in the exercise-to-exhaustion groups were run until exhaustion at 35 m/min and 0% grade. Exhaustion was defined as the point at which the rat refused to run on the treadmill, despite continual prodding by electrical

shock (3). The LCt_{50} for PFIB in rats has been determined to be in the range of 1100-1300 mg-min/m^3 (1). We chose 1100 mg-min/m^3 to be the concentration administered to animals used in the serial sacrifice experiment, and we chose 1000 mg-min/m^3 to be our sub LCt_{50} concentration in the exercise portion of the study.

Generation and Measurement of Exposure Concentrations:

PFIB was obtained from Armageddon Chemical Co, Durham, NC, at a purity of 98.5%. The relevant physical properties were as follows: an odorless colorless gas with a boiling point of 7°C and vapor density of 8.2 g/l.

The exposure concentrations were generated by using a series of flow regulating devices as shown in figure 1. The test material was quantitated using a Miran 80 (Foxboro Instrument Co., Foxboro, MA) gas analyzer with instrumental settings as follows: pathlength, 15.75 m; slit width, 1 mm; reference wavelength, 5.099 μm; sample wavelength 8.624 μm; and reference material, dry air. The Miran 80 was calibrated by injection (Model 1002, Hamilton Co., Reno, NC) of microliter volumes of the neat test gas into the closed loop volume of 5.64 liters. The calibration standards ranged from 14 mg/m^3 (10 μl) to 100 mg/m^3 (70 μl), with the absorbance vs. concentration plot nearly linear over this range.

Apparatus for Whole-Body Exposure:

Whole-body exposures of rats were done in a cylindrical 20 liter glass and plexiglas chamber. The test gas entered the chamber through multiple inlets near the base of the chamber where the test animals were located. Animals were exposed in groups of six. To retain a reasonable concentration-rise time within the inhalation chamber, the system flow was 40 lpm.

The experimental animals were placed in the inhalation chamber with fresh air flowing for 10 minutes prior to exposure. The exposure was started by switching to the challenge line and stopped 10 minutes later by returning to fresh air. Animals were kept in the chamber for 10 minutes after the end of exposure.

Observation of the Animals, Sacrifice and Tissue Collection

Serial Sacrifice:

At predetermined intervals, the rats were sacrificed by intraperitoneal injection of T-61. The lungs were surgically removed and immediately placed into Zenker's formol-saline

solution. After fixation for a minimum of 12 to 16 hours, the lungs were post-fixed in 2% potassium dichromate, trimmed, washed in running water overnight and, along with the formalin-fixed sections of heart, liver and kidney, processed by routine methods for paraffin-embedded, H&E stained sections.

Effect of Exercise:

Following exposure and exercise, rats were observed a minimum of every 2 hours for a period of 24 hours; during this time, subjective observations were made with regard to the general conditions of the groups and individual animals within the groups. At the end of the 24-hour period, the animals were euthanized by decapitation and the heads placed immediately into liquid nitrogen. The thoracic cavity was opened and the contents of the cavity to include the trachea were removed. The heart and major vessels were trimmed from the lung and the lung and caudal half of the trachea weighed. Following weighing, the right apical lobe was removed, weighed and placed in formol-saline (Zenker's) fixative. The heart and portions of the liver and kidney were placed in 10% buffered formalin. The lungs less the right apical lobe were placed on pre-weighed aluminum foil dishes and placed in a desiccating oven (Boekel Corp., Philadelphia, PA) at 100°F. Each sample was weighed daily until no further decrease in weight could be detected.

Statistics:

The data from the exercise study were compared by two-way analysis of variance (ANOVA), utilizing the BMDP statistical software program #9V (University of California Press). Data are expressed as a mean + standard error of the mean (SEM) with significance at $p < 0.05$. ANOVA tables are provided with most figures.

RESULTS

Serial Sacrifice:

Significant antemortem alterations associated with effects of the test material were limited to the lungs. Although the early lesions were minimal to mild in severity, pulmonary perivascular edema and eosinophilic perivasculitis primarily affecting venules and small veins were seen at 5 minutes post-exposure. The severity of the lung edema increased to moderate to severe in most animals by 12 hours post-exposure. Alveolar edema was prominent when lung edema was graded as moderate or severe. The severity of perivasculitis was proportional to the

cellularity of the perivascular inflammatory exudate. Acute alveolitis, first apparent at 24 hours, was characterized by noticeable leukocytic inflammatory cells in the airspace along alveolar walls, and increased leukocytes within the vessels and interstitium of alveoli. Fibrin and eosinophilic amorphous material (edema) were usually present in the inflamed alveoli. Subacute alveolitis was usually diagnosed in rats killed at 7 days post-exposure. In addition to leukocytes, fibrin and edema, epithelial cells and macrophages were present in alveoli during the subacute phase. Markedly thickened alveolar walls were also present during the subacute phase. In rats diagnosed as having chronic interstitial pneumonitis, airspaces were usually clear of cellular exudate and fibrin. Alveolar walls were thickened due to epithelial cell proliferation, mononuclear cell exudation and fibrillar material suggestive of collagen in the interstitium adjacent to larger blood vessels and subpleural interstitium. At 7 and 14 days post-exposure, perivascular inflammatory cells were still prominent. These included lymphocytes and macrophages, with a few eosinophils. For the purpose of comparison, pathology descriptions were converted to a numerical scale; the mean score for each of these conditions by group and time of sacrifice are shown in table 1.

Effect of Exercise

Endurance Time:

Figure 2 shows the results of time to exhaustion measurements for the 24 exercised animals. The 12 rats that received exposure to PFIB and exercised 30 minutes post-exposure ran for an average of 64.73 + 4.11 minutes while 12 sham control animals ran to exhaustion in 53.29 + 3.87 minutes (mean $\pm$ SEM).

Clinical Observations:

Immediately following exhaustive exercise, both the exposed group and the non-exposed group required approximately 30 minutes to return to normal activity. During this period, animals were reluctant to move within their cages and had a rapid breathing pattern. It was not until 6 hours post-exposure that the 12 rats that had been exercised after exposure were observed to be less active than the exposed/not exercised group. The exposed/exercised animals were inactive, appeared not to want to eat nor drink and had piloerection; 2 animals also had rapid breathing. The same was the case at 10 hours post-exposure except that the 2 animals noted earlier appeared to be more lethargic and seemed to have greater difficulty breathing. These 2 animals were the only fatalities of the 24 exposed animals, one animal dying 14 hours post-exposure the other 17 hours post-exposure. The 12 animals

that had been exposed but not exercised appeared to continue to eat and drink and did not demonstrate significant clinical signs of toxicity with the exception of slightly decreased activity and moderate piloerection. All unexposed animals demonstrated normal behavior for the 24-hour period following their sham exposure.

Body Weight Measurements:

Figure 3 shows that the body weights of animals in each of the four experimental groups were not significantly different.

Lung Weight Measurements:

Figure 4 demonstrates the difference in the total lung weights between the two exposed groups and between the exposed and sham exposed groups. This difference is seen when expressed both in terms of absolute grams ($p < 0.05$) and when the lung weights are normalized to a percent of total body weight ($p < 0.05$). This same relationship is shown for the right apical lobe, which was removed and weighed prior to submission for histopathological examination (figure 5).

Figure 6 depicts the weight of the lung tissue following desiccation to constant weight for the four experimental groups. Note that when expressed in terms of absolute grams of dry lung tissue, the difference between the two exposed groups is not significant ($p < 0.079$); however, the difference between exposed and non-exposed animals is highly significant ($p < 0.0001$). When these same data are normalized to each animal's body weight (figure 7), we see that a significant difference does exist between the two exposed groups ($p < 0.05$) as well as between exposed and non-exposed groups ($p < 0.0001$). This same relationship can be seen when we consider the lung water (difference between wet and dry lung weights) depicted in figure 8. Again no difference is seen between the two sham exposure groups, while significance is found between the two exposed groups ($p < 0.05$) and between the exposed and non-exposed groups ($p < 0.0001$).

Summary of Pathological Findings:

In the two groups of exposed animals, edema and congestion were constant features in variable degrees, but most severe in the exposed/exercised group. Edema was absent in only one animal and minimal in two others. All three were from the non-exercised group. The basic pattern was diffuse septal thickening due to congestion and accumulation of proteinaceous material, with exudation of edema fluid and fibrin into alveolar air spaces. Due to the acuteness of the lesion, cellular macrophage response was minimal to mild in degree. Diffuse hemorrhage into alveoli

was a prominent feature of the exposed/exercised group, but not the exposed/non-exercised group. In the sham exposed/exercised group, only three animals showed any edema, and in two of these animals, the edema was perivascular rather than alveolar in nature. One animal in the non-exposed/non-exercised group showed mild perivascular edema.

Subacute liver and heart lesions were noted, but due to the subacute nature of the cellular response, it was determined that they were not related to the experimental agent. A minimal tubular nephrosis was present in all animals and may have been the result of the fixation method.

Table 1. Average score for descriptors of pathology at time of sacrifice for rats exposed to 1100 mg-min/m^3 of PFIB and five pathologic controls.

Group	n	Time	PE	PV	EOS	IP	HIS
1	3	5 min	1.0	1.3	2.0	0	0
2	3	30 min	1.0	2.0	4.0	0	0
3	3	90 min	1.0	2.0	2.7	0	0
4	3	4 hr	2.0	2.0	3.3	0	0
5	3	12 hr	4.0	2.3	3.3	0	0.7
6	3	24 hr	4.0	2.3	0	0	0
7	3	72 hr	0	0	0	4.0	4.0
8	11	7 days	0	0.4	0.4	3.9	1.7
9	12	14 days	0	0.2	0.3	3.0	0.5
Controls	5		0	0	0	0	0
Died	4	<24 hr	4.0	2.0	2.7	0	0

PE - pulmonary edema
PV - perivasculitis
EOS - eosinophilic infiltrate
IP - interstitial pneumonitis
HIS - alveolar histiocytosis
PE, PV, IP and HIS were graded with 0 = none, 1 = minimal, 2 = mild, 3 = moderate and 4 = severe. EOS was graded with 4 = eosinophiles as predominant cell type, 2 = eosinophiles increased but not the predominant cell type and 0 = no appreciable increase in eosinophiles. All reported values are the arithmetic mean of those animals sacrificed at that time.

DISCUSSION

The serial sacrifice portion of this study has demonstrated a pulmonary infiltration eosinophilia that occurs as early as 5 minutes following exposure to near the median lethal dose of PFIB. This eosinophilia increased over the first 12 hours and was resolved by 24 hours post-exposure. Eosinophils were noted, though not found to be predominant, in the interstitial pneumonitis of one animal sacrificed at 7 days and one animal sacrificed at 14 days.

Pulmonary edema was found as early as 5 minutes post-exposure and increased through 24 hours. No animal sacrificed at 72 hours or later demonstrated pulmonary edema. Animals sacrificed at 72 hours and later showed a marked interstitial pneumonitis, which decreased only minimally by day 14. Alveolar histiocytosis was marked at 72 hours and virtually cleared by day 14. The fibrillar material in the interstitium near large blood vessels and the pleural surfaces of the lungs is suggestive of collagen formation during healing. This finding suggests that disability secondary to pulmonary fibrosis is possible in cases of sublethal exposure to this compound. Those animals that died as a direct effect of exposure demonstrated clinical signs of pulmonary edema with expectoration of clear frothy fluid prior to death.

We have demonstrated that the acute pathology associated with inhalation of PFIB is exacerbated by exhaustive exercise. We have also demonstrated that exposure at this level causes no apparent decrement in immediate post-exposure exercise endurance. The mechanism responsible for the increased pathology was not explored in this study. Physiological studies have been conducted in an effort to delineate the mechanism of PFIB-induced pulmonary edema. Studies utilizing an anesthetized and instrumented rat model to explore the role of the cardiovascular system in the pathogenesis of PFIB-induced pulmonary edema have demonstrated that the cardiovascular system plays no role in the production of PFIB-induced pulmonary edema. Suggestions have been made that the primary site of the damage may be the pulmonary vascular endothelium. These suggestions are supported by more recent work conducted at this laboratory on awake instrumented sheep (Jaeger *et al.* Draft Report), which has shown that pulmonary microvascular pressure is not increased during the period of time that pulmonary lymph flow is dramatically increasing. These present conclusions, however, do not preclude the possibility that autonomic effects caused by exhaustive exercise may potentiate subtle cardiovascular effects and thus be responsible for the increased pathology found in the exercised animals. This possibility will be examined in future studies on awake instrumented and exercising sheep.

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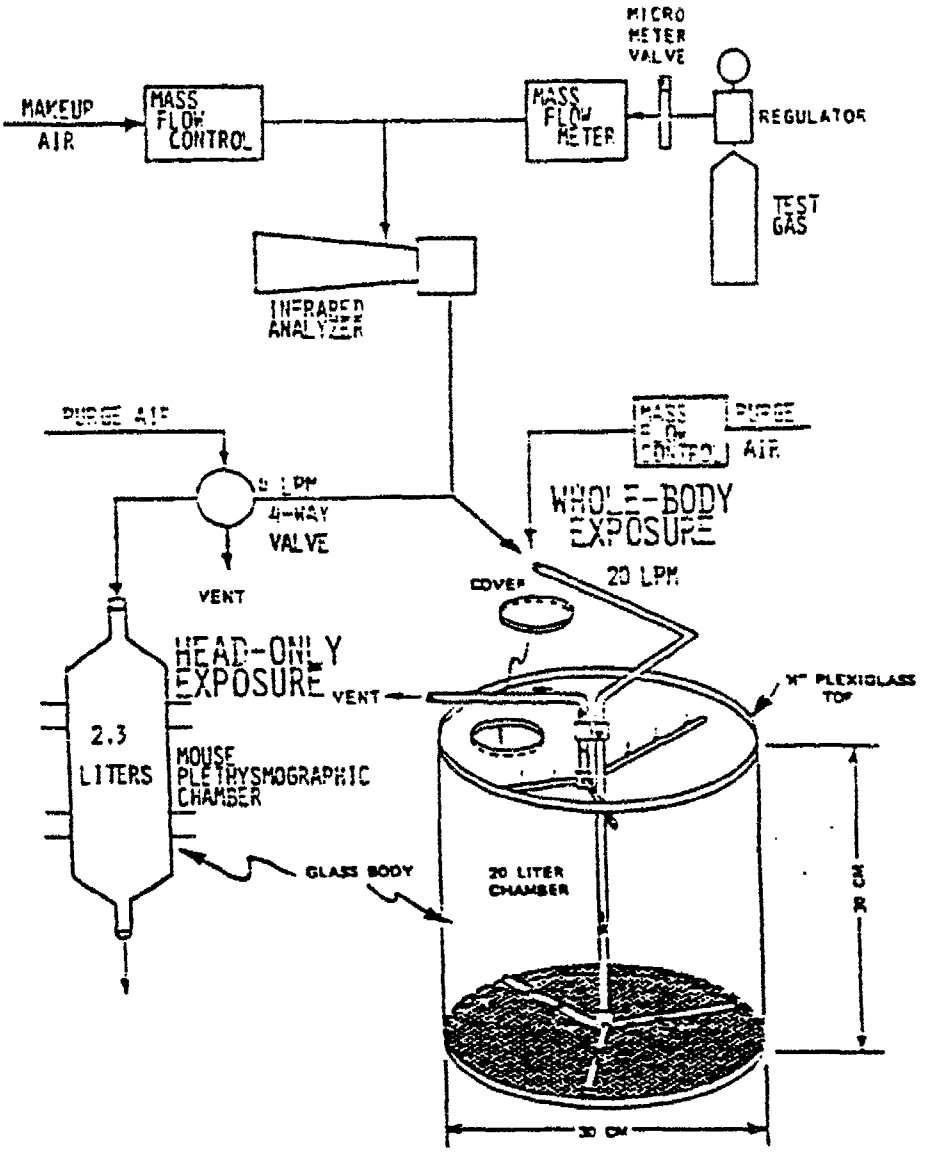


Figure 1. Apparatus used to generate specific concentrations of the test compound.

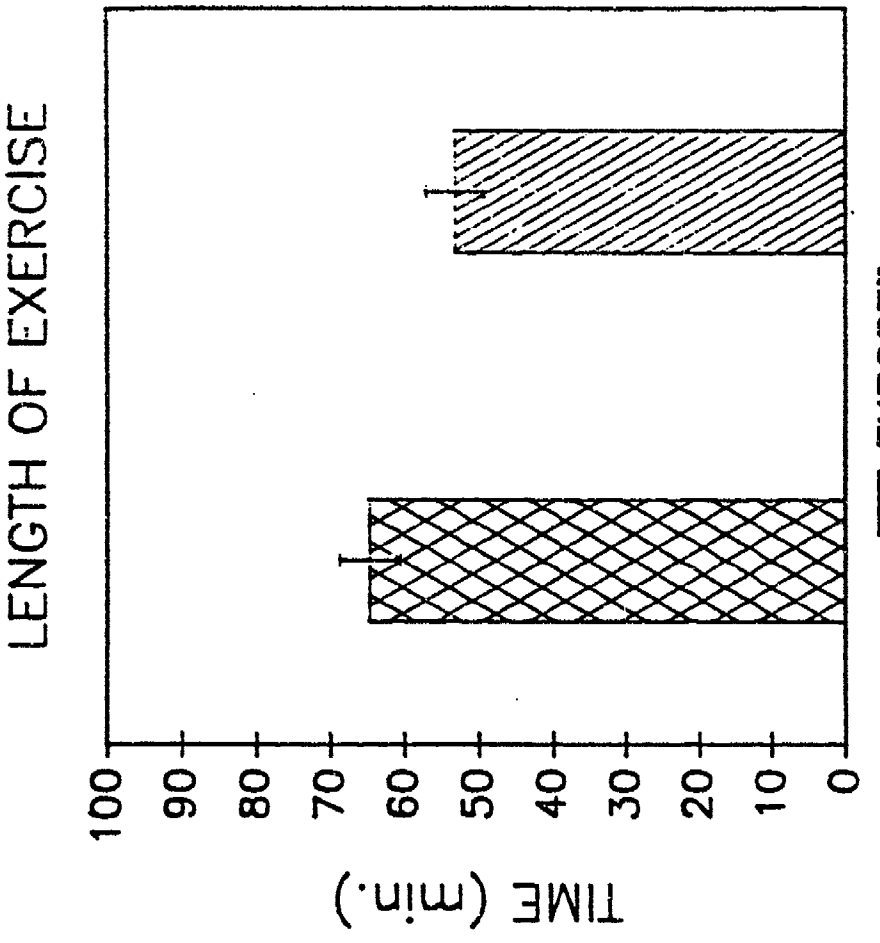


Figure 2. The effect of exposure on endurance time in rats. Two groups of treadmill acclimated rats were exercised to exhaustion and the time to reach exhaustion measured. One group was exposed to PFIB while the other group was sham-exposed. Points and bars represent mean ± SEM.

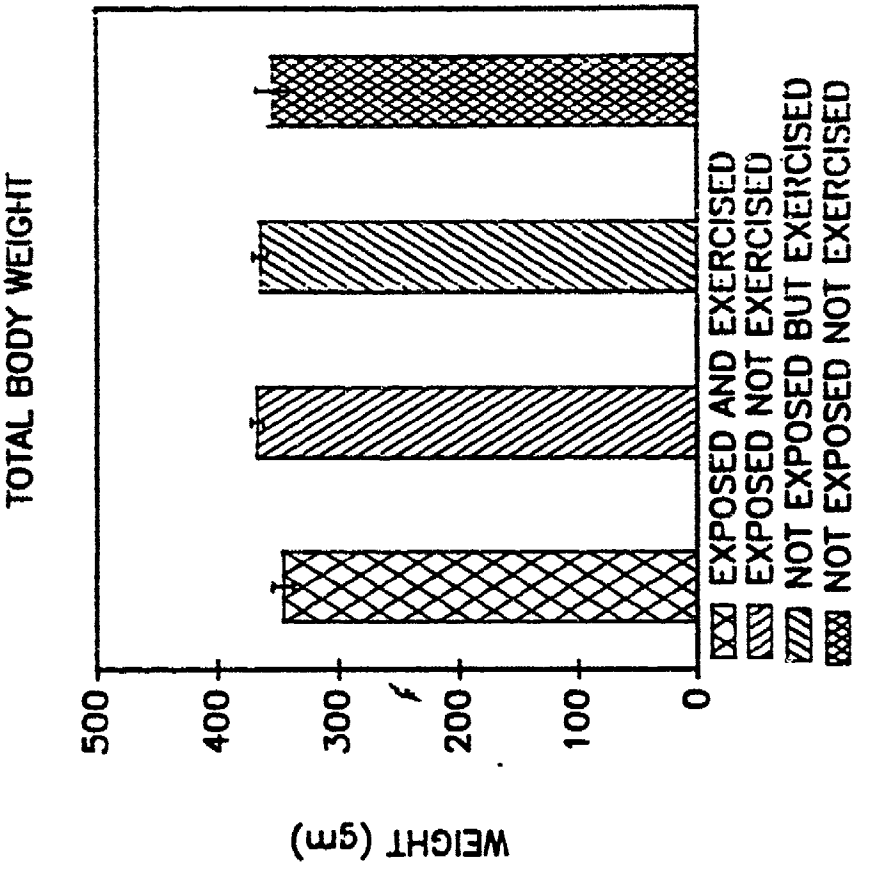
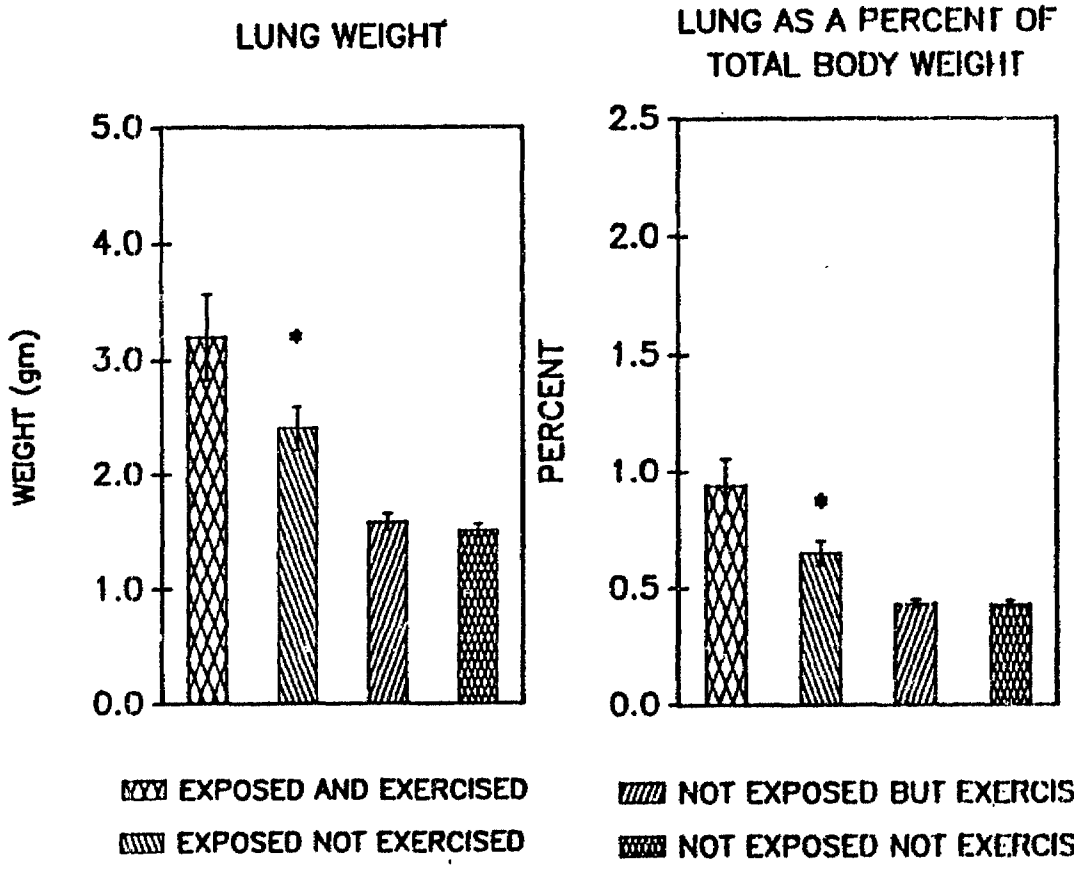


Figure 3. Body weights of 48 male Sprague-Dawley rats randomly assigned to one of four experimental groups. Points and bars represent mean ± SEM.

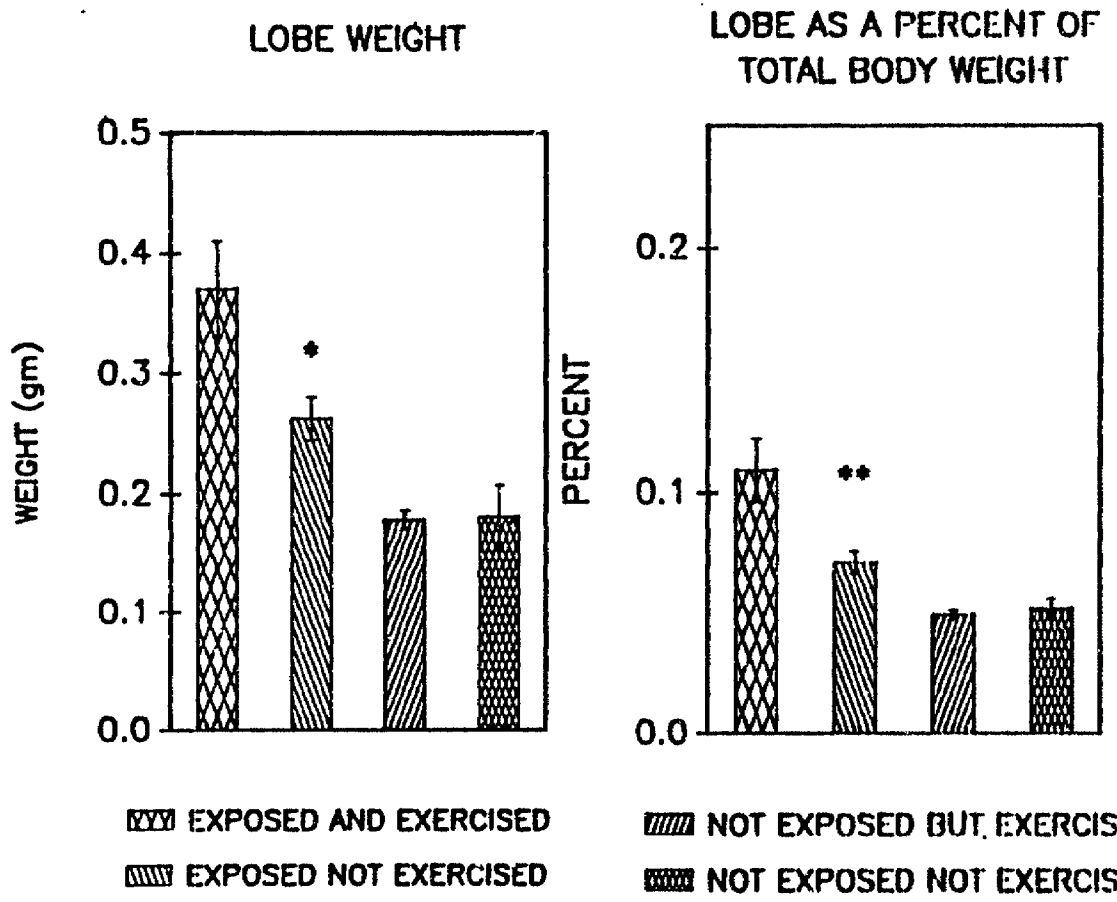


ANOVA

SOURCE	DF	F-value	p
EXPOSURE	1	31.95	0.000
EXERCISE	1	4.94	0.031
INTERACTION	1	4.68	0.036
ERROR	44		

* LESS THAN .05

Figure 4. A. Wet weight of lungs 24 hours postexposure.
B. Wet weight of lungs 24 hours postexposure expressed as a percent of each animal's body weight. Points and bars represent mean ± SEM.

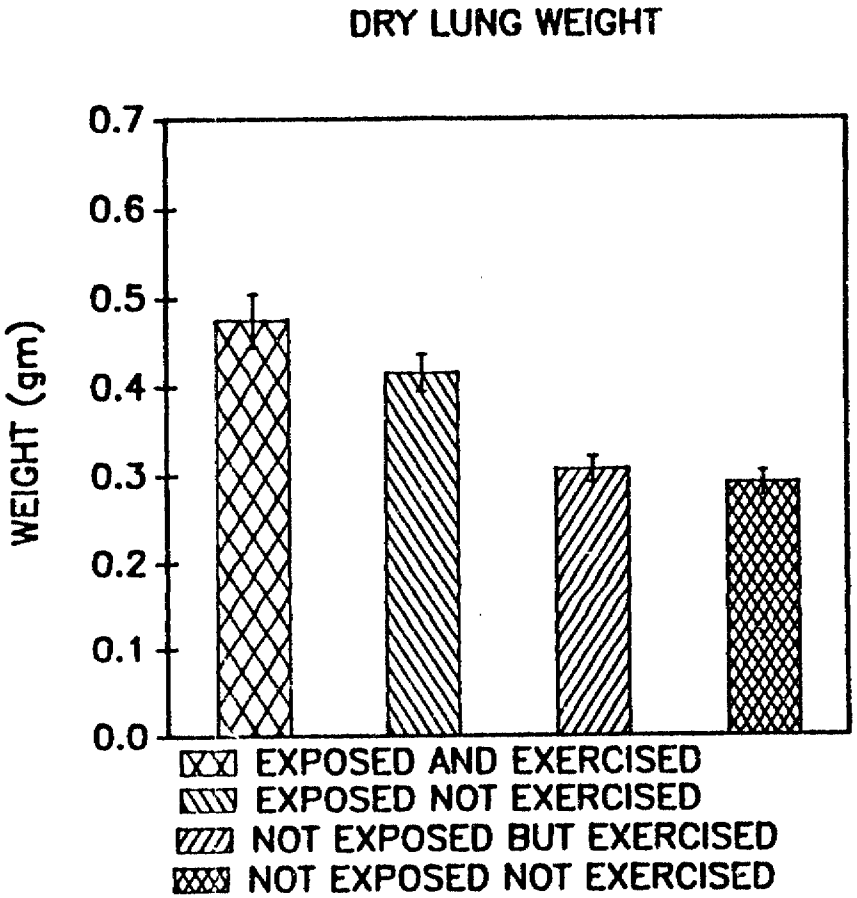


ANOVA

SOURCE	DF	F-value	p
EXPOSURE	1	29.44	0.000
EXERCISE	1	5.39	0.025
INTERACTION	1	7.78	0.008
ERROR	44		

* LESS THAN .05
** LESS THAN .01

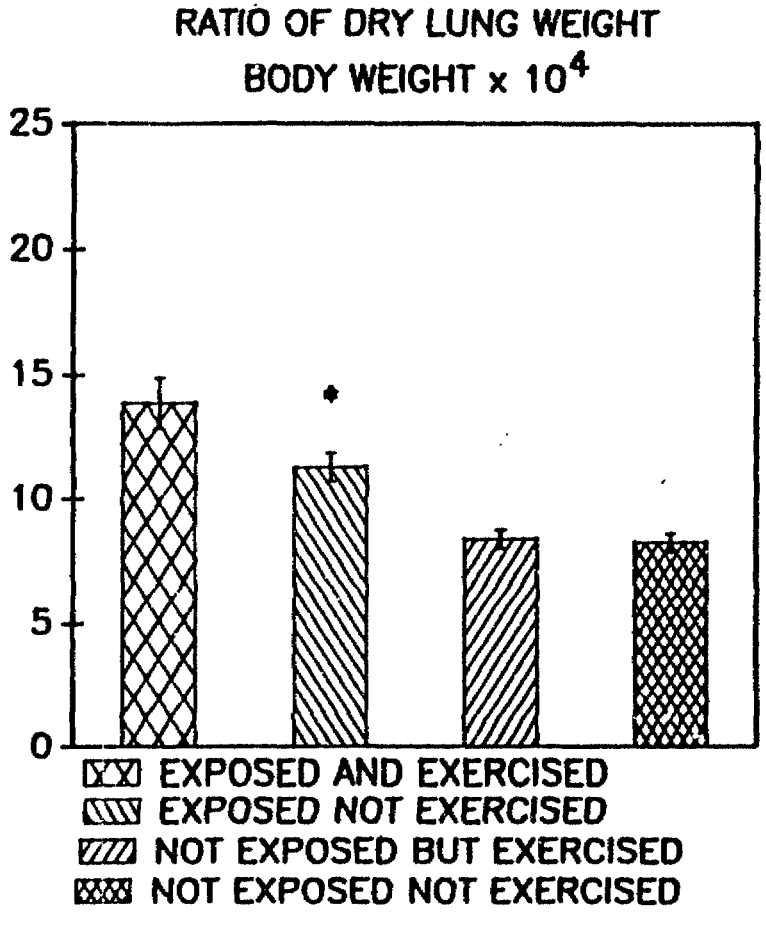
Figure 5. A. Wet weight of the right apical lung lobe 24 hours postexposure. B. Wet weight of the right apical lung lobe 24 hours postexposure expressed as a percent of each animal's body weight. Points and bars represent mean ± SEM.



ANOVA

SOURCE	DF	F value	p
EXPOSURE	1	49.28	0.000
EXERCISE	1	3.23	0.079
INTERACTION	1	1.08	0.303
ERROR	44		

Figure 6. Dry lung weights from four experimental groups following desiccation to constant weight. Points and bars represent mean ± SEM.

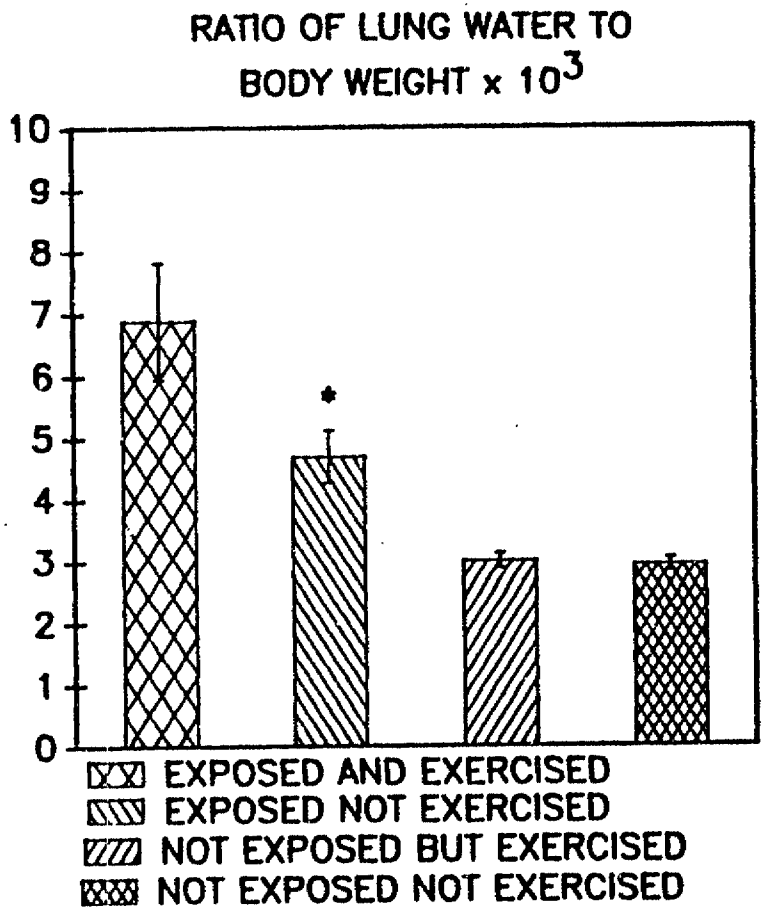


ANOVA

SOURCE	DF	F value	p
EXPOSURE	1	44.75	0.000
EXERCISE	1	4.52	0.039
INTERACTION	1	3.63	0.063
ERROR	44		

* LESS THAN .05

Figure 7. Dry lung weights from four experimental groups following desiccation to constant weight expressed as a percent of each animal's body weight. Points and bars represent mean ± SEM.

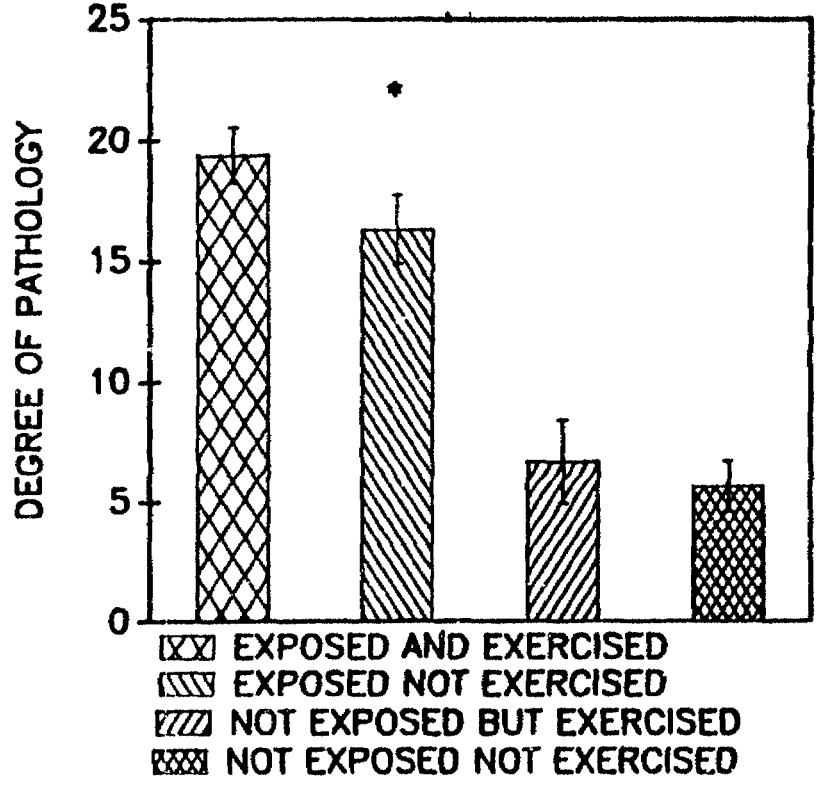


ANOVA

SOURCE	DF	F value	p
EXPOSURE	1	28.88	0.000
EXERCISE	1	4.62	0.037
INTERACTION	1	4.15	0.048
ERROR	44		

* LESS THAN .05

Figure 8. Lung water (wet weight-dry weight). Data are normalized to each animal's body weight. Points and bars represent mean ± SEM.



ANOVA

SOURCE	DF	F value	p
EXPOSURE	1	28.88	0.000
EXERCISE	1	4.62	0.037
INTERACTION	1	4.15	0.048
ERROR	44		

* LESS THAN .05

Figure 9. Degree of Pathology. The presence and distribution of edema, fibrin, congestion, hemorrhage and cellular inflammatory response were quantitated for each of the 24 animals exposed to PFIB. Points and bars represent mean ± SEM.

DISCUSSION AFTER MAJ MOORE'S PRESENTATION

As a matter of clarification, MAJ Moore indicated that the study described in his presentation was the first preliminary study that had been done. No physiologic variables other than the ones discussed were monitored. For example, changes in blood gases were not measured. However, MAJ Moore noted that histopathology was performed in livers, hearts, and kidneys (in addition to the lungs), but no lesions were found.

Dr. Mark was interested in knowing if there were any differences in the pathology of those animals that died spontaneously after exposure and those that were killed at 24 hours. In response, MAJ Moore suggested that the animals that died naturally probably had greater pulmonary congestion than the animals that were sacrificed. Mr. Harris was interested in the time course of injury. MAJ Moore responded that the time course of injury would be covered during a later session describing studies done with rats and sheep. Finally, it was noted that the exercised animals definitely displayed greater pulmonary histopathology. A grading system was used to quantitate pathology in lungs from rats exposed to PFIB; quantitative pathology could not be done in lungs from rats exposed to TFD since the lesions were so severe. MAJ Moore speculated that the cause of death after exposure to organohalides was probably asphyxiation.

Dr. Said noted that one must be cautious in interpreting the physiologic condition of animals that ran for long periods of time after exposure. He noted that some of the lung sections shown by MAJ Moore indicated that the alveolar spaces were filled with exudate, and that he [Dr. Said] found it hard to believe that these animals could function and run so well, especially when one would expect gas exchange to be compromised because of the alveolar exudate. Dr. Said suggested that, despite the fact that rats were in poor physiologic shape (in terms of the deteriorating condition of the respiratory system caused by the organohalide), they ran for longer times than they ordinarily would have because they were being driven by some sort of CNS excitation. MAJ Moore responded by saying that it was highly likely that if these animals had been allowed to run for even longer times, there would indeed have been a dramatic decrease in endurance. He clarified the design of the experiment, noting that animals started running 15 minutes after exposure; pathology, however, was not examined until 24 hours after exposure. It was suggested that at 15 minutes there probably was no major pathological change, thus explaining the similarity in the running endurance of the animals.

Finally, MAJ Moore reiterated that controls were included in these studies; control animals were exposed to the same conditions as experimentals but not submitted to endurance running.

PROGRESSIVE PULMONARY PATHOLOGY OF TWO ORGANOHALIDES IN RATS

LTC James B. Nold and Dr. John P. Petrali

The objective of this study was to evaluate the morphologic progression of the pulmonary lesions which occur after inhalation of the organohalide compounds PFIB and TFD, using light and electron microscopy. Rats were exposed to the approximate LCt_{50} dose of organohalide (100 mg PFIB/m^3 or 200 mg TFD/m^3 for 10 minutes) and sacrificed at varying time intervals (5, 30, 90 minutes; 4, 12, 24, and 72 hours) by i.p. injection of T-61. Tissues were fixed using standard methods.

Results indicated that the induced lesions were generally quite similar in PFIB- and TFD-exposed rats. Using the progression of TFD lesions as an example, striking features of the lung injuries included the following changes:

> At the light-microscopic level, minimal to mild edema was first seen 30 minutes after exposure. At 90 minutes, light microscopy revealed mild, multifocal perivascular and alveolar edema, mildly increased alveolar macrophages, and increased alveolar eosinophils. The pinnacle of organohalide-induced lung injury seemed to occur at 12 hours, when diffuse and severe edema with diffuse fibrin deposition (which suggests loss of vascular integrity and increased permeability of proteins), increased alveolar macrophages, and septal hypercellularity and thickening were observed. The extent of edema and fibrin deposition was lessened at 72 hours, indicating limited recovery, but severe thickening of the alveolar septum, hyperplasia of Type II epithelial cells, and elevated levels of alveolar macrophages were still present. Electron microscopy (EM) revealed greater details of the pulmonary injury. Vascular congestion, increased electron density and pinocytotic activity of endothelial cells, and focal vacuolation and membrane damage of Type I epithelial cells were evident as early as 5 minutes after exposure. Areas of frank necrosis were apparent at 12 hours, and at 72 hours the EM picture was characterized by hypertrophy of Type II epithelial cells, alveoli filled with exudate and mononuclear cells, and loss of cytoarchitecture.

Overall, injury due to TFD was slightly more severe than with PFIB, and progressed faster.

PROGRESSIVE PULMONARY PATHOLOGY OF TWO ORGANOHALIDES IN RATS

LTC James B. Nold, Dr. John P. Petrali,
Dr. Henry H. Wall, MAJ David H. Moore,
MAJ Kenneth G. Phillips, and LTC Nancy K. Jaax

U.S. Army Medical Research Institute of Chemical Defense
Pathophysiology Division
Aberdeen Proving Ground, Maryland

OBJECTIVE

Evaluate the progressive pulmonary morphologic changes associated with an inhaled organohalide compound at the light-microscopic and electron-microscopic levels.

EXPERIMENTAL DESIGN

- 48 age-, weight-, and sex-matched rats exposed (whole body) to a constant flow of 100 mg/m^3 perfluoroisobutylene (PFIB) for 10 minutes
- 21 age-, weight-, and sex-matched rats exposed (whole body) to a constant flow of 200 mg/m^3 bis-(trifluoromethyl) disulfide (TFD) for 10 minutes
- Euthanatized in groups of 3 by IP T-61 at the following observation times:

  5 minutes/30 minutes/90 minutes
  4 hours/12 hours/24 hours/72 hours

- 9 (5/4) unexposed rats served as controls
- Tissues fixed in Zenker's and 4CF-1G fixatives for light and electron microscopy

SUMMARY - LIGHT MICROSCOPY

PROGRESSIVE PULMONARY PATHOLOGY - TFD

- 5 Minutes
  - Normal
- 30 Minutes
  - Minimal to mild edema (Zenker's)
- 90 Minutes
  - Mild, multifocal perivascular and alveolar edema
  - Mildly increased alveolar macrophages
  - Increased perivascular eosinophils
- 4 Hours
  - Diffuse, moderate perivascular and alveolar edema
  - Moderately increased alveolar macrophages
  - Mild septal thickening
  - Increased perivascular eosinophils and neutrophils
- 12 Hours
  - Diffuse, severe edema
  - Diffuse, moderate fibrin deposition
  - Increased alveolar macrophages
  - Septal hypercellularity and thickening
- 24 Hours
  - Diffuse, severe, edema, and fibrin
  - Septal thickening and epithelial hypertrophy
  - Increased alveolar macrophages
- 72 Hours
  - Less edema and fibrin
  - Severe septal thickening and epithelial hyperplasia
  - Increased alveolar macrophages

SUMMARY - ELECTRON MICROSCOPY

PROGRESSIVE PULMONARY PATHOLOGY - TFD

- 5 Minutes
  - Vascular congestion
  - Increased electron density and pinocytotic activity of endothelial cells
  - Focal vacuolization and membrane damage of type I epithelial cells
- 30 Minutes
  - Mild to moderate interstitial edema
  - Focal vacuolization of type I epithelial cells
  - Loss of lamellar bodies of type II epithelial cells
  - Active macrophagic activity
- 90 Minutes
  - Widening of alveolar septa
  - Interstitial edema
  - Increased vacuolization and pinocytotic activity of type I epithelial cells
  - Blebbing and rupturing of type I plasmalemma
  - Recruitment of type II cells with active endoplasmic reticulum
- 4 Hours
  - Severe irregularities of the plasmalemma of type I cells with invaginations and blebbing
  - Rarefaction of type II cell cytoplasm
  - Increased loss of type II cell lamellar bodies
  - Alveolar edema with fibrin deposition
- 12 Hours
  - Increased vacuolization and blebbing of type I cells
  - Hemorrhage with alveolar fibrin deposition
  - Areas of frank necrosis
- 24 Hours
  - Disrupted cytoarchitecture
  - Hemorrhage with increased amounts of fibrin deposition
  - Septal basal lamina denuded of type I cells
- 72 Hours
  - Hypertrophy of type II epithelial cells
  - Alveoli filled with exudate and mononuclear cells
  - Loss of cytoarchitecture

DISCUSSION AFTER LTC NOLD AND DR. PETRALI'S PRESENTATION

Several points were clarified in this discussion. First, this effort was not designed to include a temporal evaluation of the effects of exercise; in the previously discussed exercise study, there was only a single sacrifice time point after exercising the animals. Second, this study was not designed to allow detection of early changes in vascular smooth muscle tone. It was commented that effects on cell proliferation were seen only after 72 hours. Finally, it was noted that the time course for changes in epithelium and endothelium was about the same.

Discussion continued, focusing on the possible mechanisms responsible for the pathological changes observed. The question was raised as to whether the progression of injury after the organohalides was similar in any fashion to the injury seen after smoke or acid inhalation. No consensus was achieved regarding similarity to the effects of smoke. It was believed, however, that there is probably more primary airway epithelial damage after acid inhalation than after administration of organohalides. LTC Nold stated that with TFD and phosgene at the gross level, there was more mucous exudate filling the airways than with PFIB. It was his opinion that TFD is more of an irritant to epithelial tissue. Dr. Petrali said he believed that, regardless of the initial insult, there is probably a common injury pathway consisting of a cascade of events resulting in tissue damage.

Dr. Lehnert noted the lack of polymorphonuclear leukocytes in the micrographs shown by LTC Nold and Dr. Petrali; he considered this to be curious since their presence is usually a hallmark of acute lesions resulting from edematous injuries. LTC Nold pointed out that infiltration of PMNs is usually not seen in the rat, and that in sheep there is generally only a minor infiltration of neutrophils into the alveoli [in response to lung injury caused by these compounds].

Dr. Said expressed the opinion that infiltration of cells would be seen only after clinical infections. Dr. Lehnert however, disagreed, saying that the infiltration of the PMNs was clearly the result of inflammatory injuries. Dr. Fowler said that smoke inhalation is characterized by profound neutrophil influx across the alveolar interstitium into the airways. Dr. Lehnert suggested that it is important to follow the mechanism of injury, and noted that it is possible that free radicals aren't involved as a component of the injury mechanism itself. LTC Jaax commented that it is necessary to distinguish between cytotoxic injury and inflammatory injuries which involve mononuclear cells. The possibility was raised that at early time

points (4-12 hours), only resident alveolar macrophages were being observed, while at 22 hours there was clearly a monocyte influx.

Additionally, it was suggested by Dr. Lehnert that perhaps part of the leak at the alveolar level was actually the result of retrograde translocation of fluid from conducting airways. That is, the fluids seen peripherally may actually have been aspirated from the airways. The question was then raised as to whether the investigators actually looked at conducting airways for epithelial damage. LTC Nold and Dr. Petrali responded that this had not been observed at the light-microscopic level.

MAJ Ripple was interested in discussing the chemistry of organofluorines; asking: (1) whether these compounds were highly lipid soluble; and (2) whether they were directly cytotoxic as opposed to being irritants. He felt this was important since it may be possible that organofluorines are entering the cells directly and ultimately causing cell death, as opposed to exerting their effects from outside of the cell, that is, on the cell membrane. In addition, it was asked whether the effects of organofluorines were at all similar to the effects of plant phytogens in regard to their ability to cause pulmonary edema. These questions were not resolved.

Dr. Werrlein and Dr. Petrali thought it possible that endothelial cell relaxing factor is involved in the endothelial cell effects seen in the micrographs.

BEHAVIORAL EFFECTS FOLLOWING MULTIPLE EXPOSURES TO TWO ORGANOHALIDES IN RATS

Dr. Suzanne B. McMaster

The objective of this study was to determine if multiple sublethal exposures to PFIB or TFD influenced conditioned behaviors. Rats were trained to perform an operant task (a lever-press paradigm) under a multiple-time-out fixed-ratio schedule of reinforcement (using food as the reinforcement). Submitted to head-only exposure, rats received increasing doses of organohalide at intervals of 1 week to several months. The dose of PFIB ranged from 100 to 1700 mg-min/m^3 and the dose of TFD ranged from 300 to 2000 mg-min/m^3; these represented the 0.1-1.5 LCt_{50}. Behavioral manifestations were evaluated 2 and 24 hours after exposure.

Neither PFIB nor TFD affected baseline behavior at moderate doses. Effects were seen only after high doses: at 1300 mg-min/m^3, PFIB caused approximately a 25% decrease from baseline; and for TFD, the dose of 2000 mg-min/m^3 caused about a 40-50% decrement. At these high doses, rats did not appear healthy, being visibly affected by the organohalides. However, since the compounds did not have a clear dose-dependent effect on behavior, and since profound effects were seen only at the highest doses, it was concluded that organohalides have no significant toxic effect in the CNS.

BEHAVIORAL EFFECTS FOLLOWING MULTIPLE EXPOSURES TO TWO ORGANOHALIDES IN RATS

Dr. Suzanne B. McMaster, MAJ Kenneth G. Phillips,
Anita V. Finger, and SGT Felix Feliciano

U.S. Army Medical Research Institute of Chemical Defense
Pathophysiology Division
Aberdeen Proving Ground, Maryland

PURPOSE

Evaluate potential behavioral effects of multiple sublethal exposures to PFIB or TFD

APPROACH

- Rats were trained to perform an operant task under a multiple-time-out, fixed-ratio schedule of reinforcement
- Exposed to increasing concentrations of an organohalide at week or more intervals
- Exposures were head only: concentrations ranged from 0.1-1.5 LD_{50}

  100-1700 mg-min/m^3 PFIB

  300-2000 mg-min/m^3 TFD
- Behavior was evaluated at 2 and 24 hours post-exposure

RESULTS

Figures and tables follow.

CONCLUSION

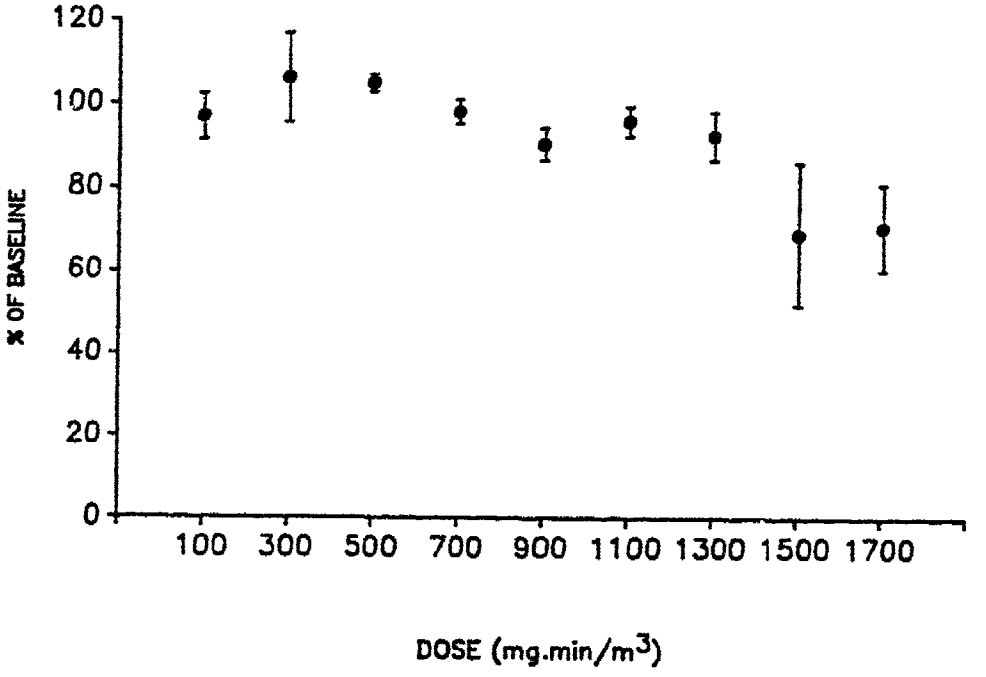
The organohalide compounds tested have no direct toxic effect on the CNS.

Results:

PFIB n =		
DOSE	+ 2 HOURS	+ 24 HOURS
100	3	3
300	3	3
500	3	3
700	10	10
900	10	10
1100	10	10
1300	10	10
1500	7	4
1700	4	0

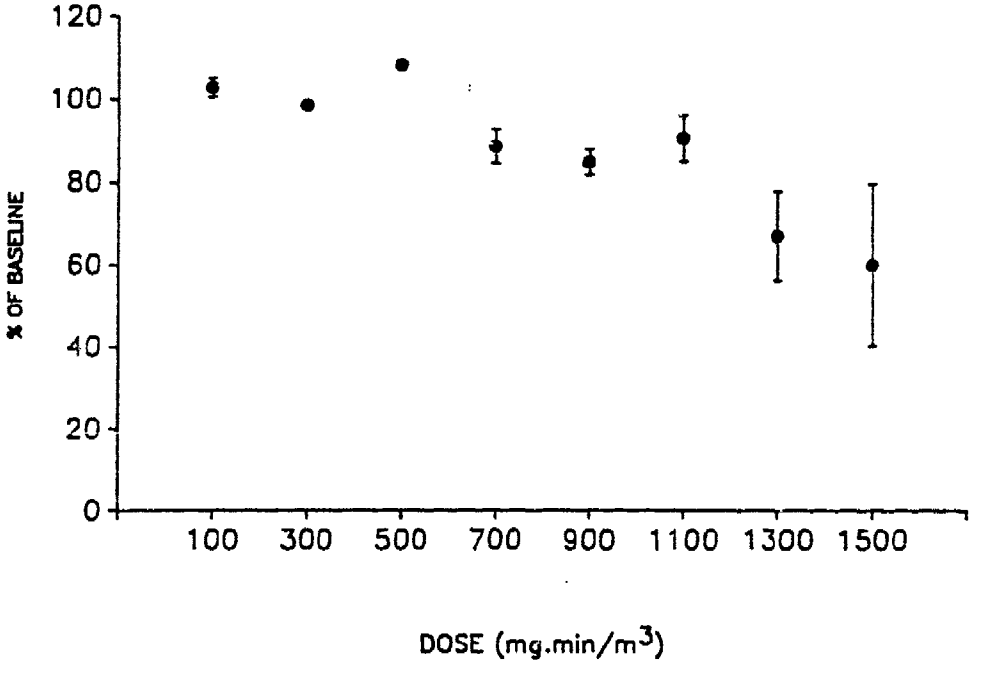
PFIB

EXPOSURE + 2 HOURS

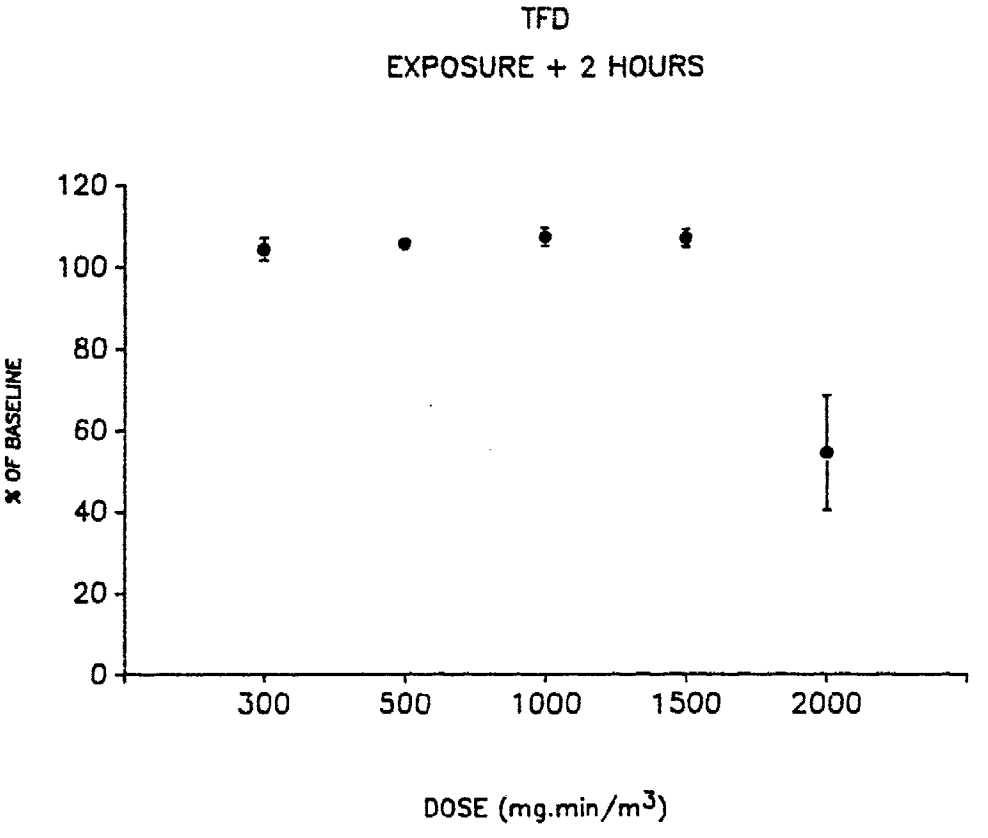


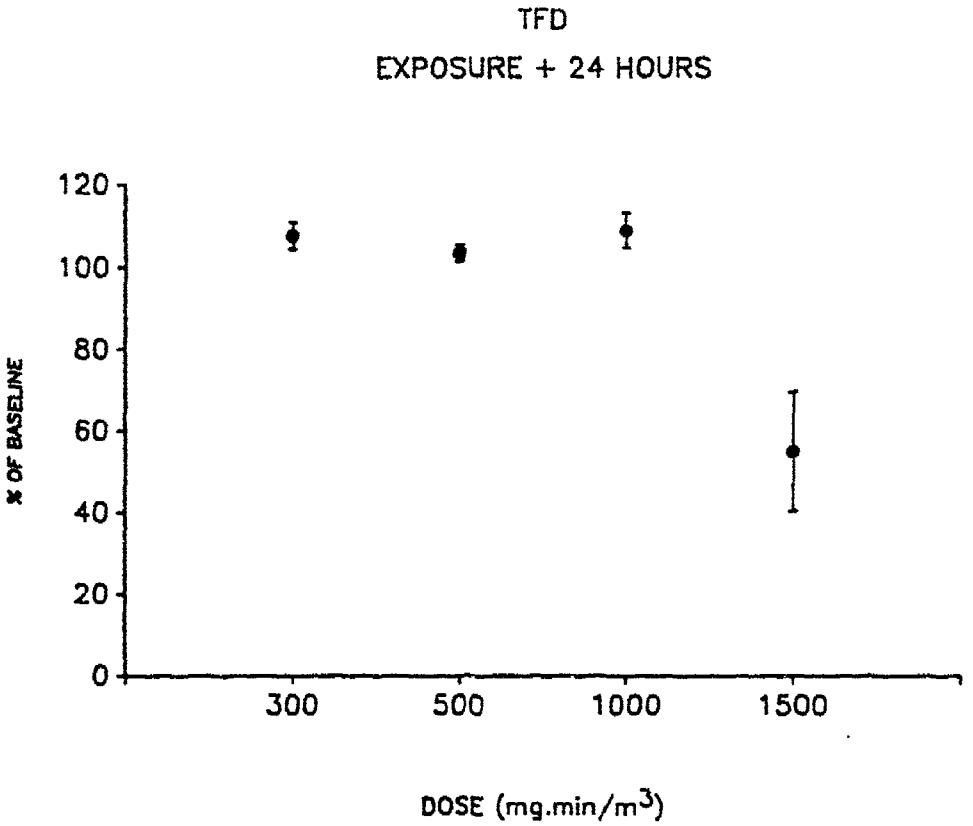
PFIB

EXPOSURE + 24 HOURS



TFD n =		
DOSES	+ 2 HOURS	+ 24 HOURS
300 - 1500	11	11
2000	6	0




TFD
EXPOSURE + 24 HOURS
120
100
80
60
40
20
0
% OF BASELINE
300
500
1000
1500
DOSE (mg.min/m3)

DISCUSSION AFTER DR. McMASTER'S PRESENTATION

LTC Keeler and Dr. McMaster discussed the fact that there may have been some cumulative effects of the drug since, although the LD_{50} was thought to be 2000 mg, all of the rats died at this dosage. LTC Jaax described the response as basically an "all or none phenomenon," and commented that there seemed to be a characteristic response in those rats receiving the lower dose: lower-dose rats displayed less pulmonary edema but more lymphocyte infiltration as compared to rats that died from a single high dose. LTC Jaax added that one problem in this study was that they did not have normal-aged controls with which to compare the treated animals.

Dr. Lehnert brought up the subject of whether animals develop tolerance to repeated dosages. MAJ Phillips and Dr. McMaster commented that there was evidence of this with PFIB. It was further commented that there may have been problems with organohalide deposition into tissues when animals were given repeated low-dose exposures [which resulted in the cumulative effect discussed above].

Dr. Kramer questioned whether these drugs can cross the blood-brain barrier, and it was generally agreed that they do. It was noted that in rats, the only observed effects of these compounds has been pulmonary failure with no evidence of central deficits.

ORGANOHALIDE-INDUCED PULMONARY EDEMA* AND EFFECTS OF PHOSGENE** IN A CHRONICALLY INSTRUMENTED SHEEP MODEL

*LTC James J. Jaeger and **LTC Jill R. Keeler

The objective of these efforts was to develop an animal model for the study of pulmonary edema. Once established, such a model can be used to evaluate compounds that cause pulmonary edema, such as the organohalides, and further, to examine the efficacy of interventions on various physiologic parameters.

The clinically instrumented sheep model involved the cannulation of the efferent duct of the caudal mediastinal lymph node. (In general, lymph is believed to closely describe the content of the interstitial fluid compartment.) This technique would allow the study of lymph flow rates, volume changes, and protein content. Thus, overall fluid flux could be continually monitored. Other surgical maneuvers allowed the measurement of arterial pressure, heart rate, and blood gases and the calculation of left atrial pressure (LAP) and pulmonary artery pressure (PAP). Surgical preparation of the sheep usually required 5-7 hours; animals were then allowed to rest for 3-5 days before experimentation. Sheep were submitted to nose-only exposure, with a cycle of 10 minutes of breathing air--10 minutes of test compound--10 minutes of air. Data were collected 3-30 hours after exposure.

Exposure to PFIB at a dose of 20-25 g/m^3 over a 10-minute period resulted in a striking four- to fivefold increase in pulmonary lymph flow, and an increase in the ratio of lymph/plasma proteins. No significant effect was seen on arterial pressure, arterial blood gases and pH, heart rate, PAP or LAP. Sheep died of asphyxiation 4-5 hours after exposure. It was concluded that PFIB induced permeability edema.

Exposure to TFD at a dose of 30,000-50,000 $mg\text{-}min/m^3$ resulted in a significant increase in pulmonary lymph flow; no significant effects were observed in the other variables measured. It was concluded that TFD also induced permeability edema, and the mechanism of TFD was probably different from that of PFIB since lymph/plasma protein ratio was not greatly affected by TFD.

Exposure to phosgene at a dose of 20-25 g/m^3 over a 10-minute period resulted in approximately a threefold increase in lymph flow. There was no change in the lymph/plasma protein ratio. A small increase in microvascular pressure was recorded, and this event may have contributed to the development of edema.

In summary, the sheep lung-lymph model has a demonstrated utility for the study of the effects of inhalants and physiologic interventions. In these experiments, both PFIB and TFD caused permeability-type pulmonary edema. It was not possible, however, to directly compare the dose-response to PFIB and TFD because sheep were breathing spontaneously (i.e., they were unanesthetized and not ventilated) and tended to hold their breath during TFD exposure. Finally, it was found that lymph flow measurements can predict edema long before the overt signs and symptoms of agent intoxication become apparent.

ORGANOHALIDE-INDUCED PULMONARY EDEMA IN A CHRONICALLY INSTRUMENTED SHEEP MODEL

MAJ James J. Jaeger, Dr. Holcombe H. Hurt,
MAJ Kevin D. Corcoran, John H. Parrish,
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U.S. Army Medical Research Institute of Chemical Defense
Aberdeen Proving Ground, Maryland

PHYSIOLOGY BRANCH
SPECIAL PROJECT

OBJECTIVES OF SHEEP STUDIES

- Establish animal model for the study of pulmonary edema
- Determine type of pulmonary edema caused by compounds of interest
- Determine effects of interventions on physiologic parameters

FLUID MOVEMENT IN THE ALVEOLUS (alv.)

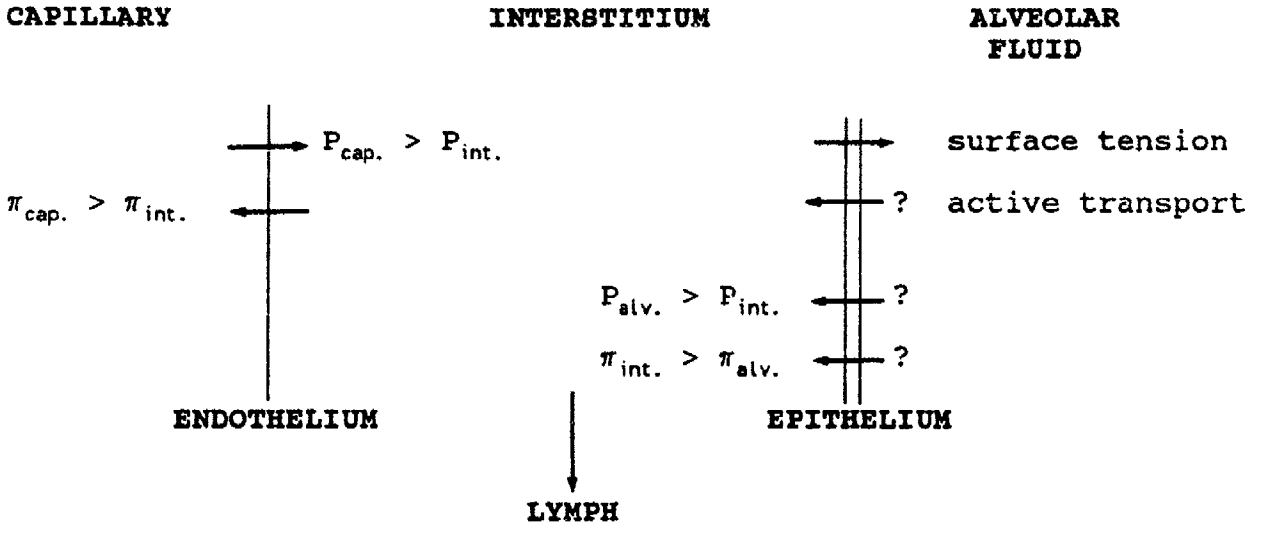


Diagram of forces for fluid movement across the endothelium and epithelium. (Adapted from Mason *et al.*, 1983. Am. Rev. Respir. Dis. 127:S24-S27.) $P_{cap.}$ and $P_{int.}$ are the hydrostatic pressures in the capillaries and interstitium, respectively. $\pi_{cap.}$ and $\pi_{int.}$ are the capillary and interstitial proteins and osmotic pressures, respectively.

SURGICAL PREPARATION AND PARAMETERS MEASURED

- Efferent duct of caudal lymph node
  Lymph flow and protein content
- Carotid artery
  Arterial pressure and heart rate
  Blood gases and pH
  Plasma protein content
- Left atrium
  Left atrial pressure
- Pulmonary artery
  Pulmonary artery pressure
  Wedge pressure
  Cardiac output
- 3-5 days of recovery
  Body temperature
  Lymph flow

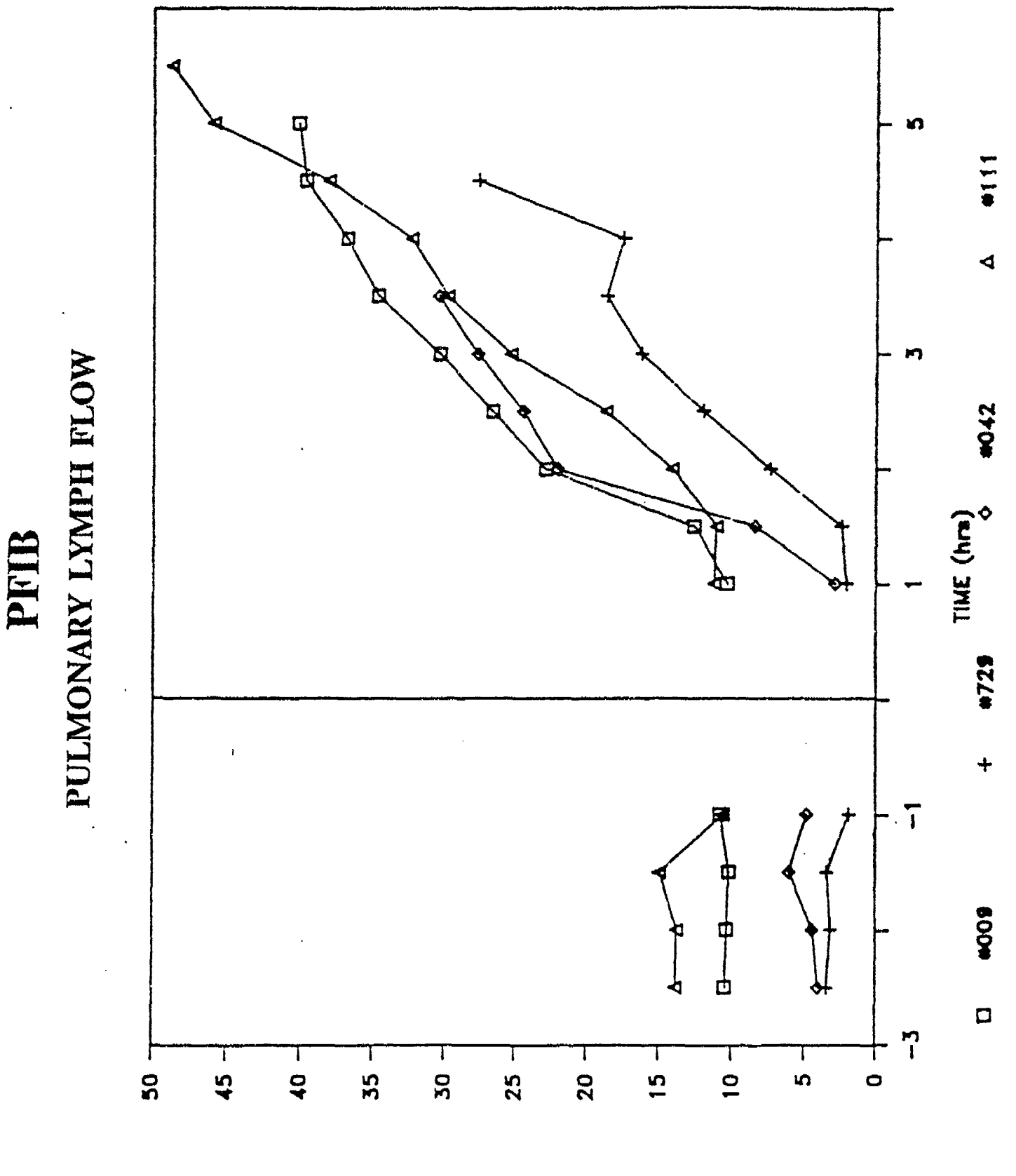
EXPERIMENTAL DESIGN

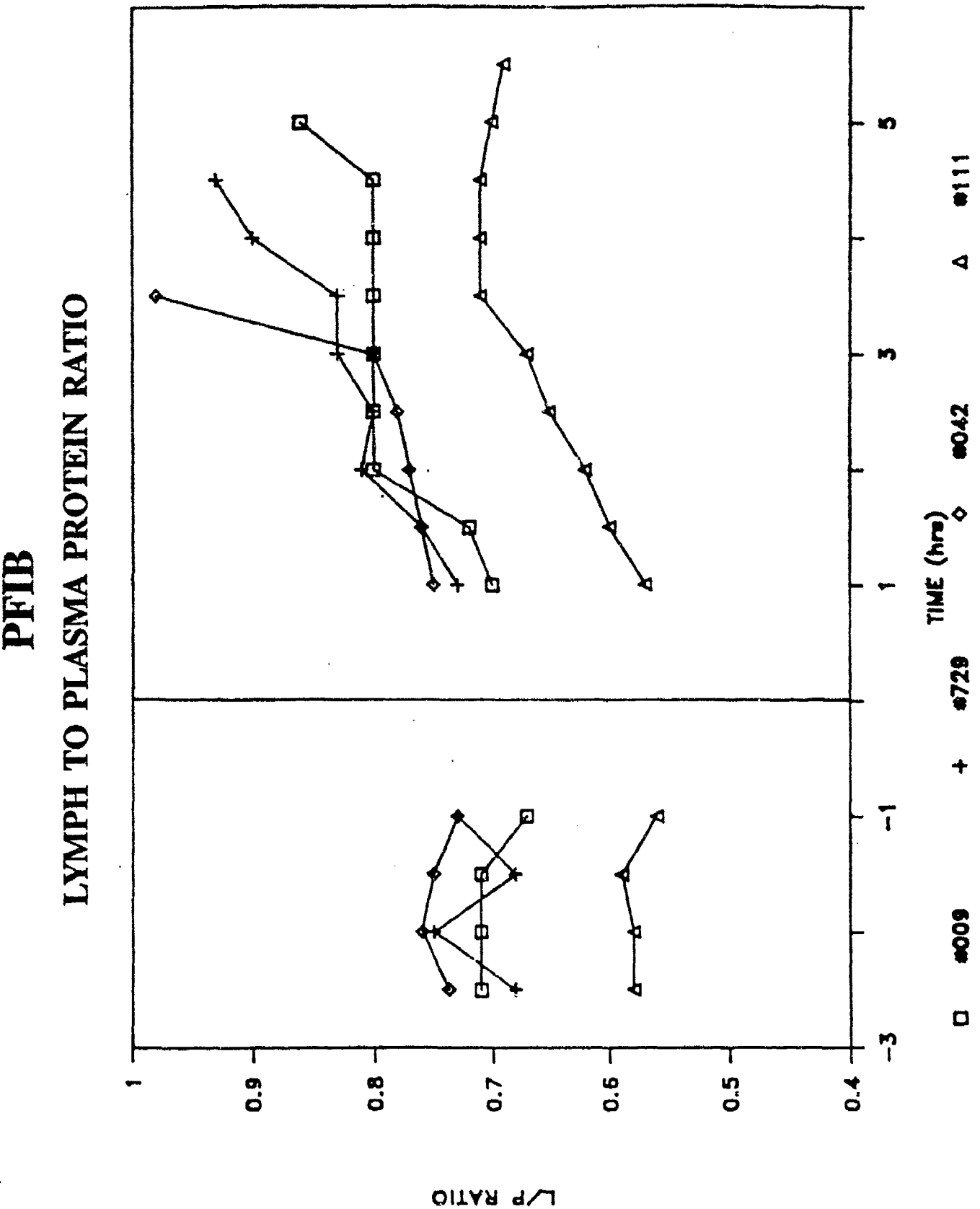
- Surgical preparation (5-7 hours)
- 3-5 days recovery
- Transport to exposure site
- Nose-only exposure
  - 10 minutes breathing air
  - 10 minutes breathing test compound
  - 10 minutes breathing air
- Return to laboratory
- 3- to 30-hour postexposure data collection

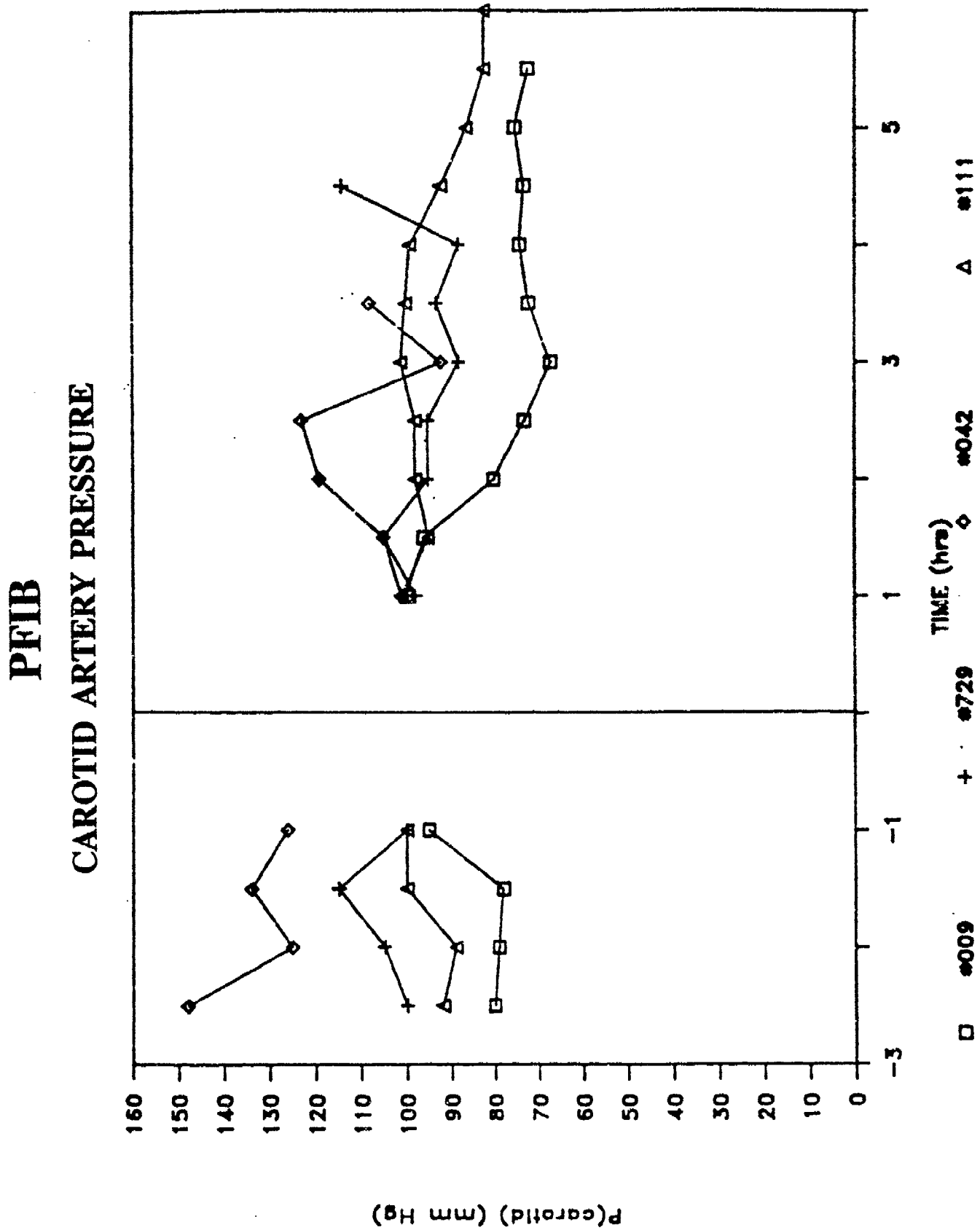
PHYSIOLOGY BRANCH
SPECIAL PROJECT

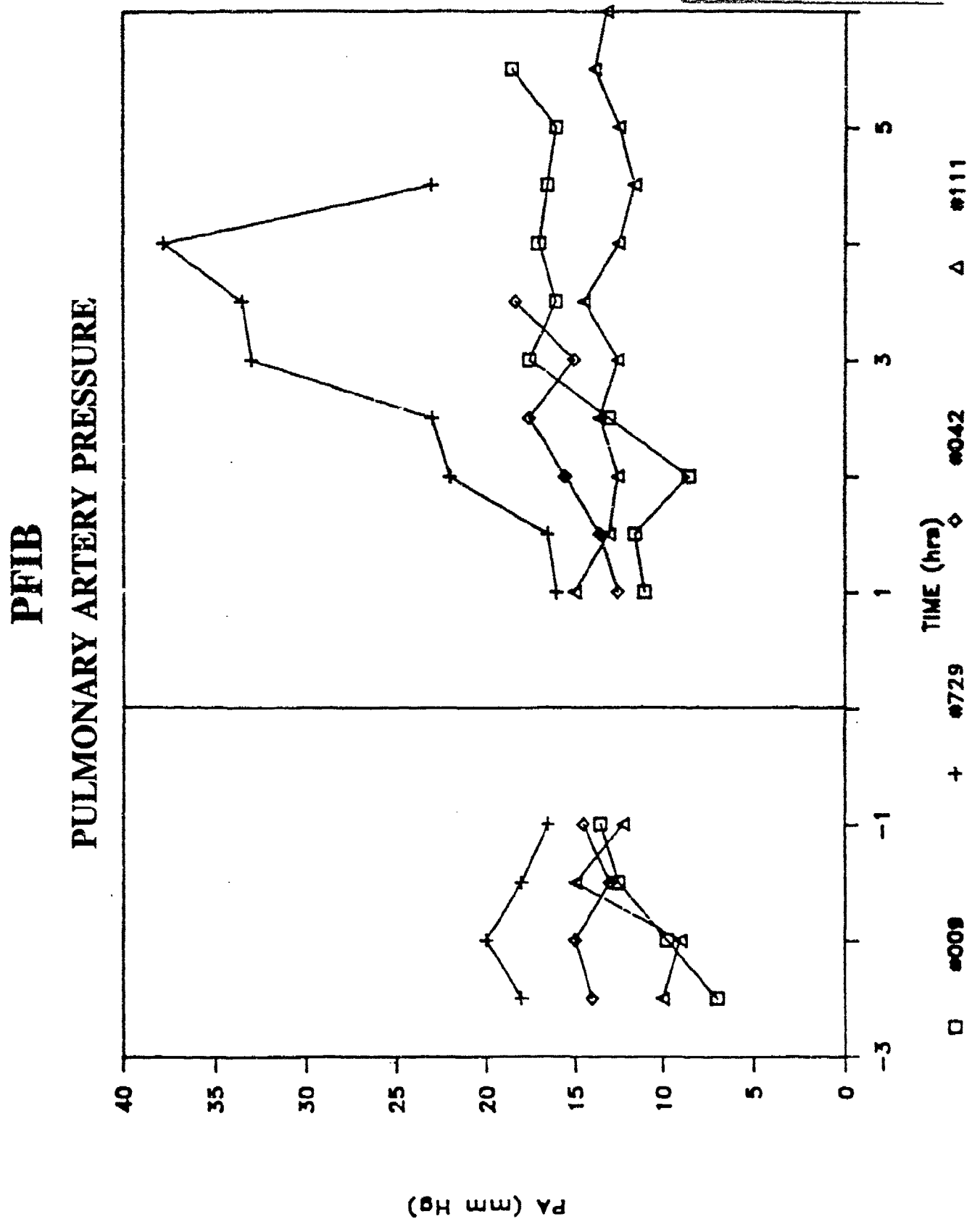
PFIB: 4 SHEEP, MEAN BODY WEIGHT - 35 KG

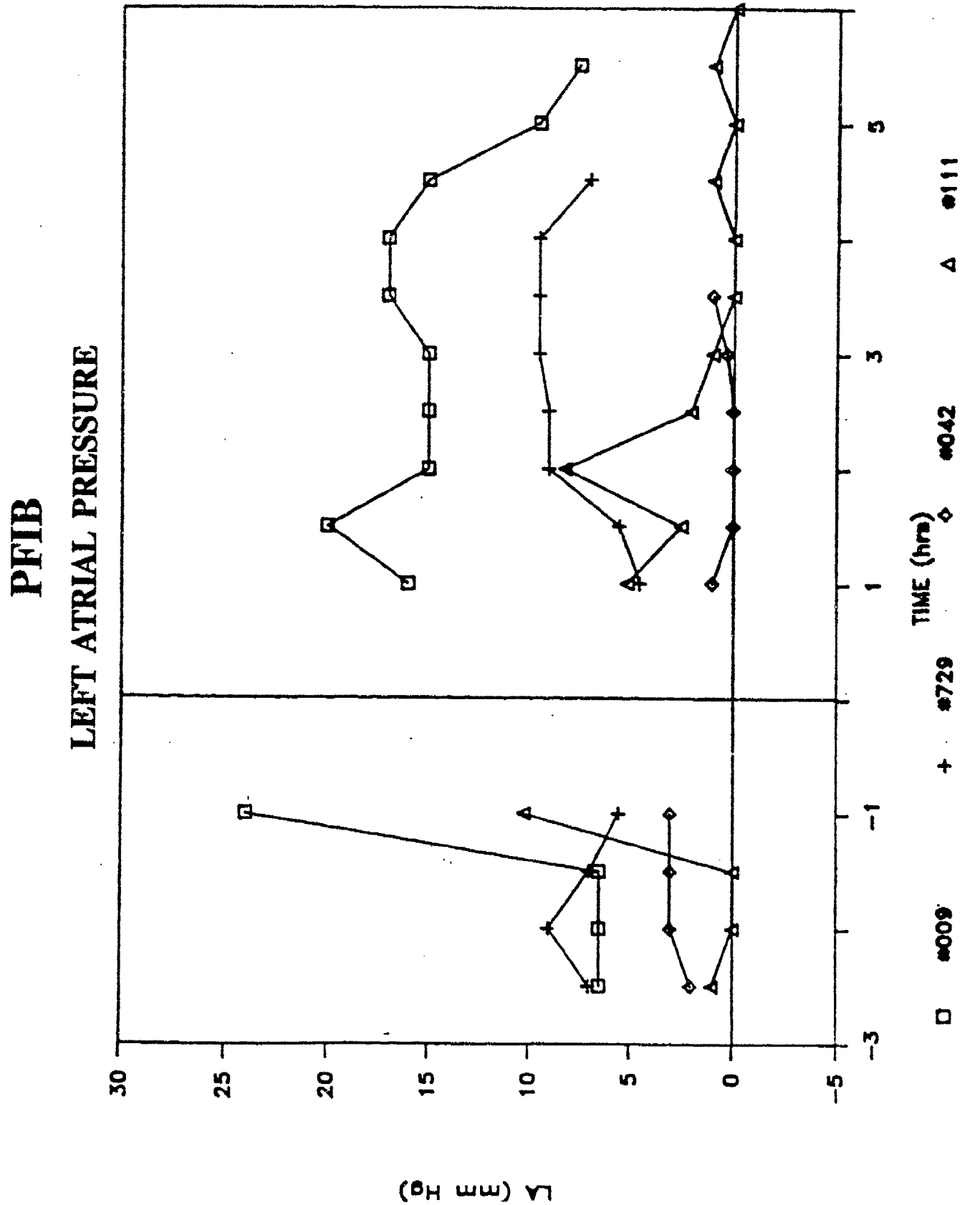
- Ct = 20,000 mg-min/m^3
- No data on Ve or blood gases during exposure
- Postexposure: No effect on
  - Arterial blood pressure
  - Arterial blood gases or pH
  - Heart rate
  - Pulmonary artery pressure
  - Left atrial pressure
- Postexposure: Significant effect upon
  - Pulmonary lymph flow
  - Lymph/plasma protein ratio
- **Conclusion:** Permeability edema


PFIB
PULMONARY LYMPH FLOW
LYMPH FLOW (gm/hr)
50
45
40
35
30
25
20
15
10
5
0
-3
-1
1
3
5
TIME (hrs)
□ #009
+ #729
◇ #042
△ #111


PFIB
LYMPH TO PLASMA PROTEIN RATIO
L/P RATIO
1
0.9
0.8
0.7
0.6
0.5
0.4
-3
-1
1
3
5
TIME (hrs)
□ #009
+ #729
◇ #042
△ #111

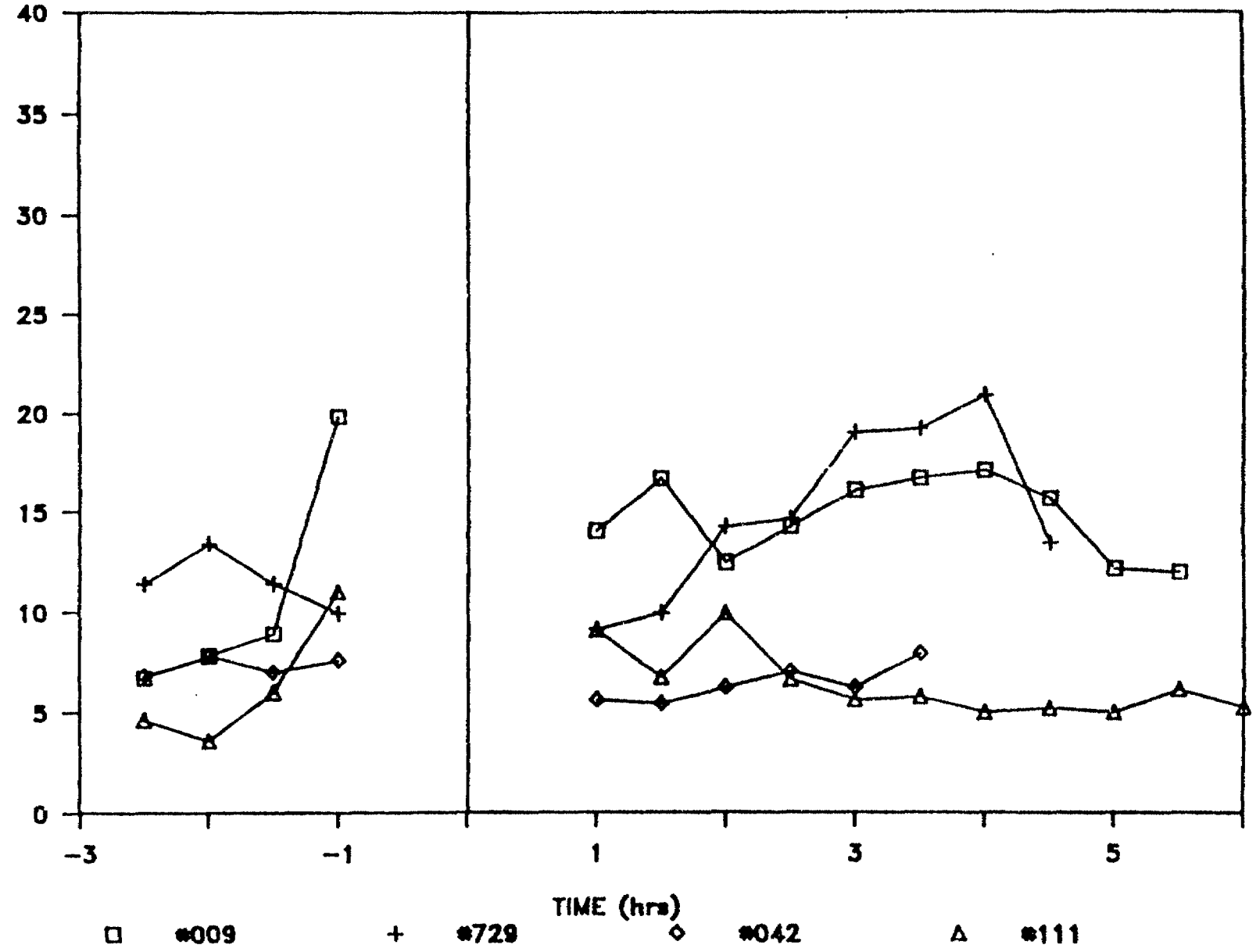

PFIB
CAROTID ARTERY PRESSURE
P(carotid) (mm Hg)
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-3
-1
1
3
5
TIME (hrs)
□ #009
+ #729
◇ #042
△ #111


PFIB
PULMONARY ARTERY PRESSURE
PA (mm Hg)
40
35
30
25
20
15
10
5
0
-3
-1
1
3
5
TIME (hrs)
□ #009
+ #729
◇ #042
△ #111


PFIB
LEFT ATRIAL PRESSURE
LA (mm Hg)
30
25
20
15
10
5
0
-5
-3
-1
1
3
5
TIME (hrs)
□ #009
+ #729
◇ #042
△ #111

PFIB

PULMONARY MICROVASCULAR PRESSURE

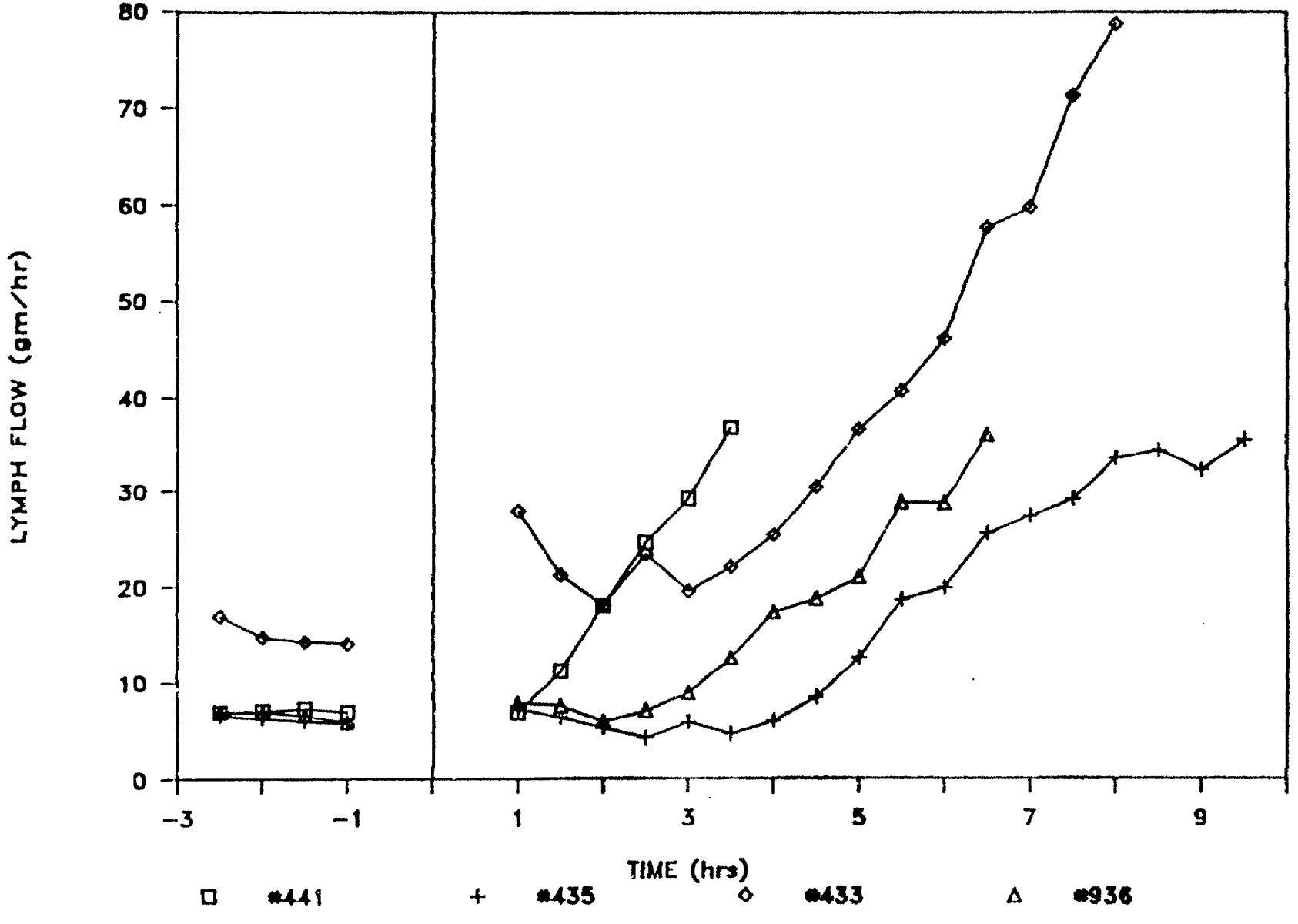


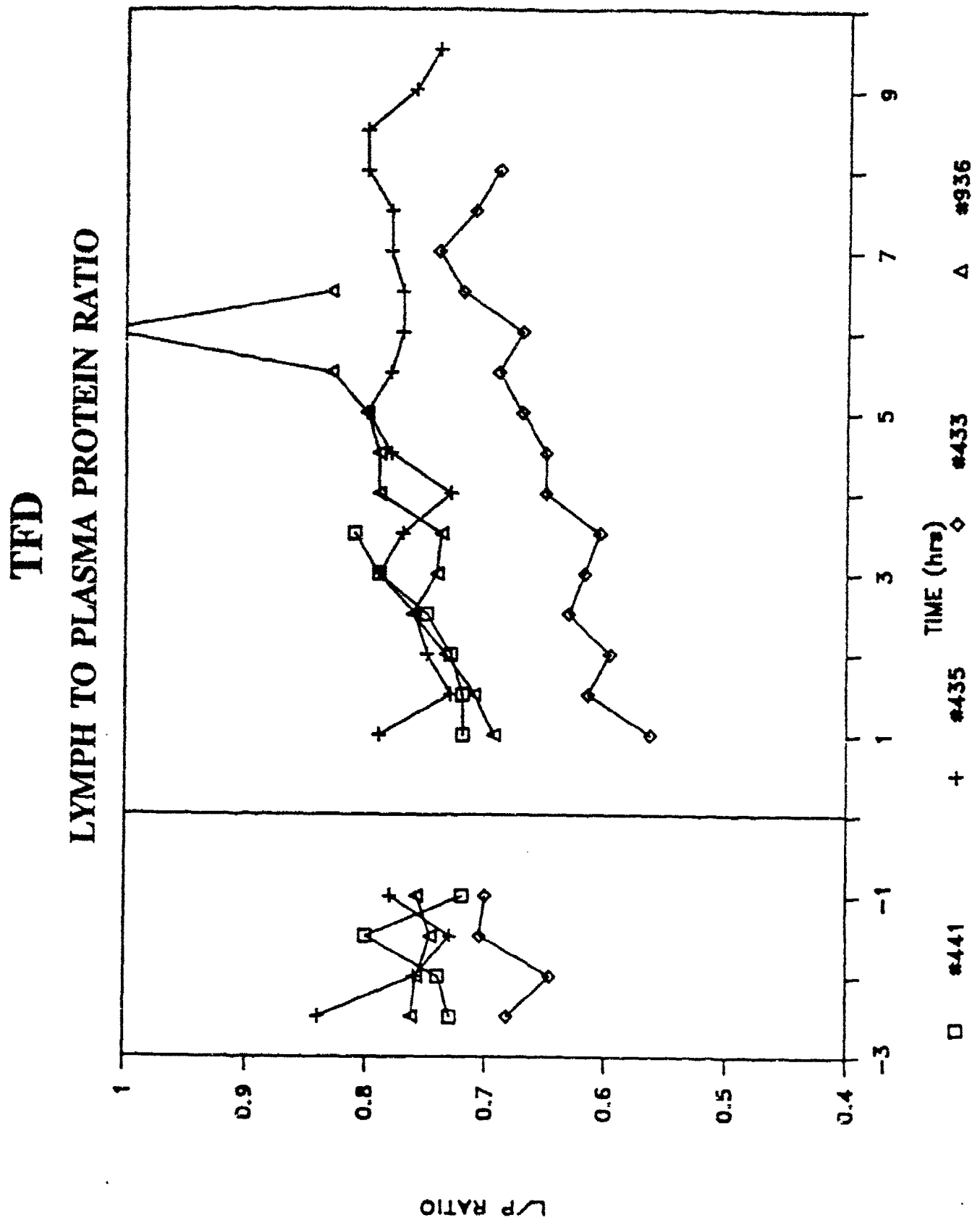
TFD: 4 SHEEP, MEAN BODY WEIGHT - 44 KG

- Ct = 30,000-50,000 mg-min/m^3
- Significant breath-holding during exposure
- Postexposure: No effect on
  - Arterial blood pressure
  - Arterial blood gases and pH
  - Cardiac output and heart rate
  - Pulmonary artery pressure
  - Left arterial pressure
  - Lymph/plasma protein ratio
- Postexposure: Significant effect on
  - Pulmonary lymph flow
- **Conclusion:** Permeability edema

TFD

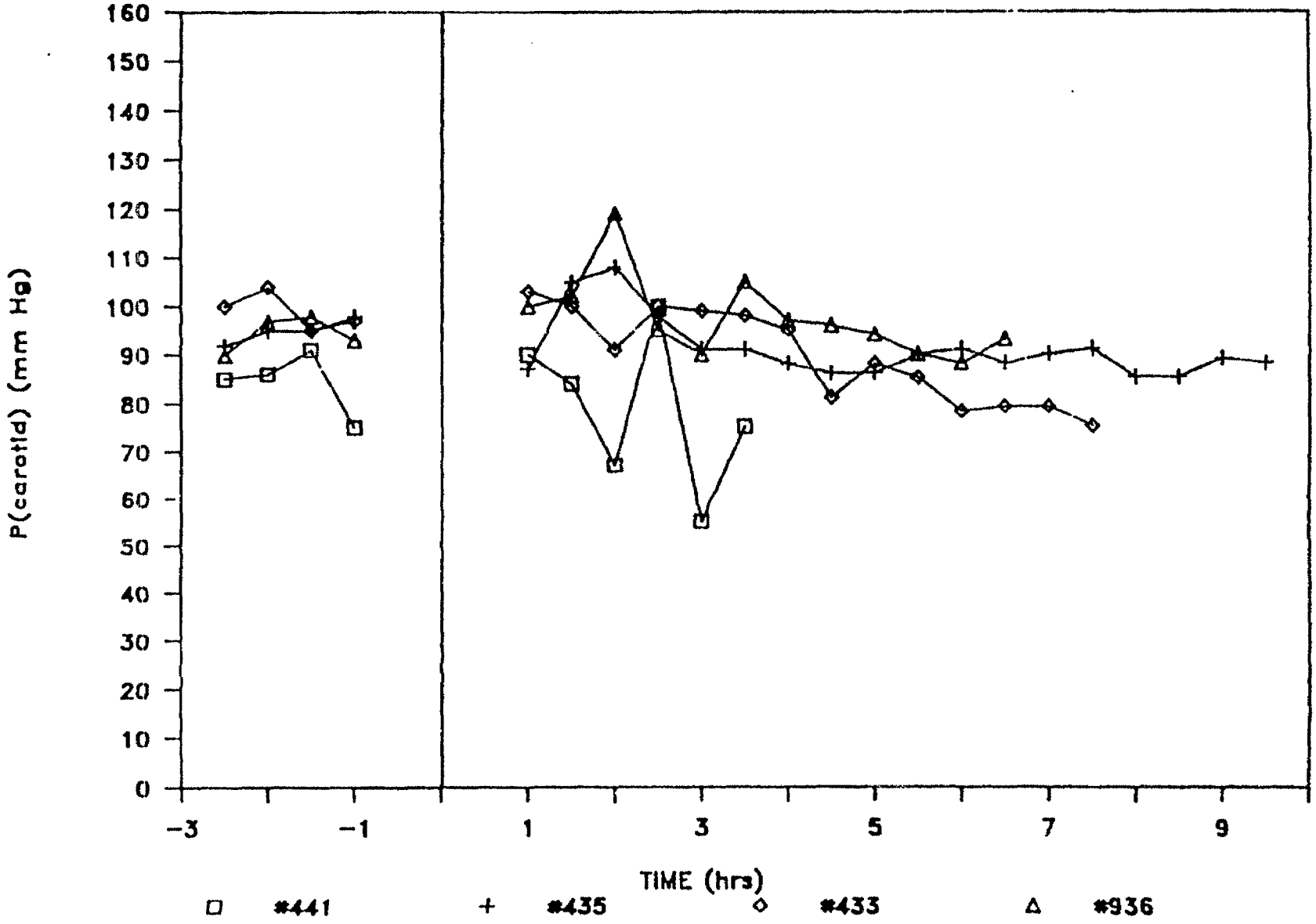
PULMONARY LYMPH FLOW

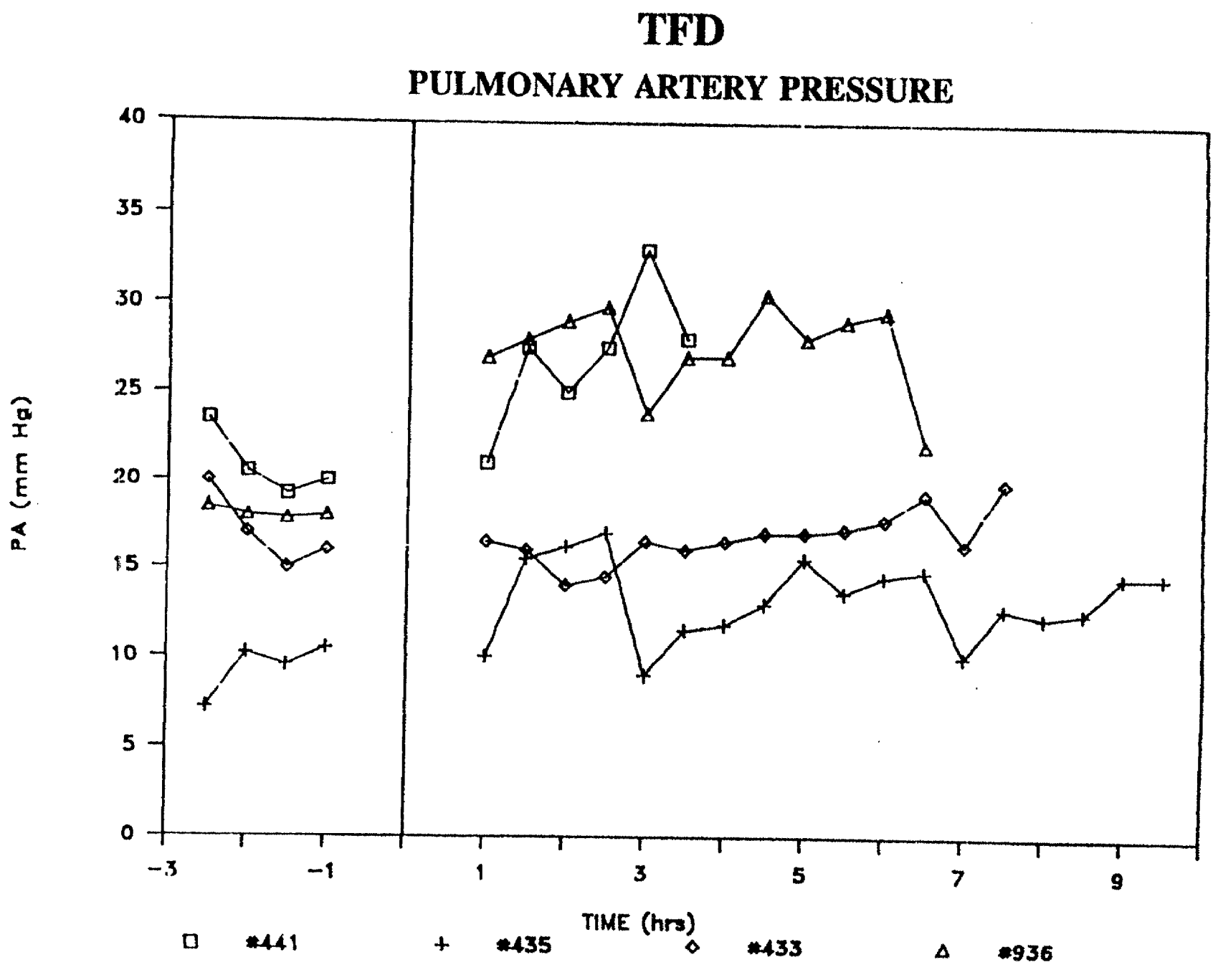


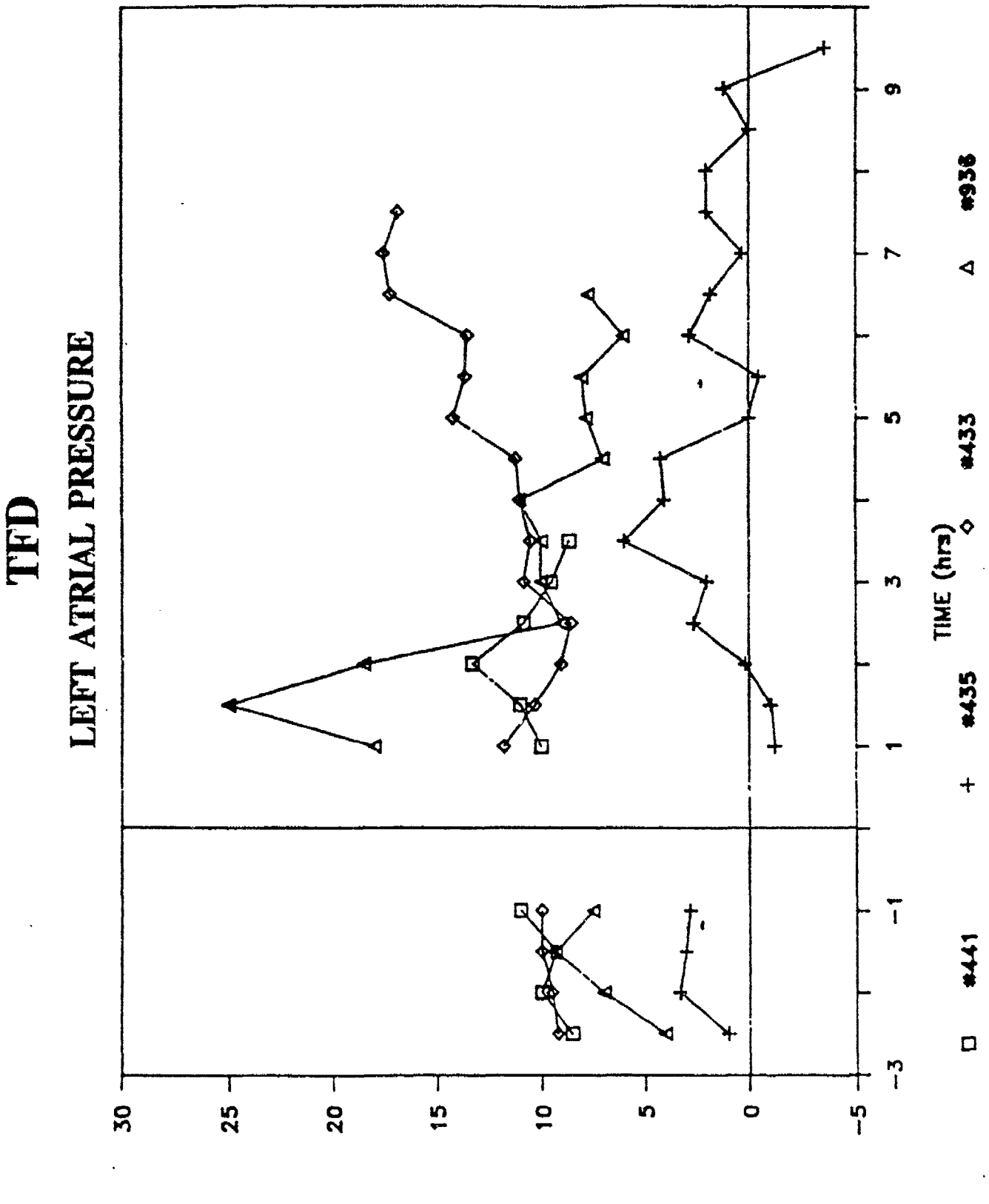

TFD
LYMPH TO PLASMA PROTEIN RATIO
L/P RATIO
1
0.9
0.8
0.7
0.6
0.5
0.4
-3
-1
1
3
5
7
9
TIME (hrs)
□ #441
+ #435
◇ #433
Δ #936

TFD

CAROTID ARTERY PRESSURE

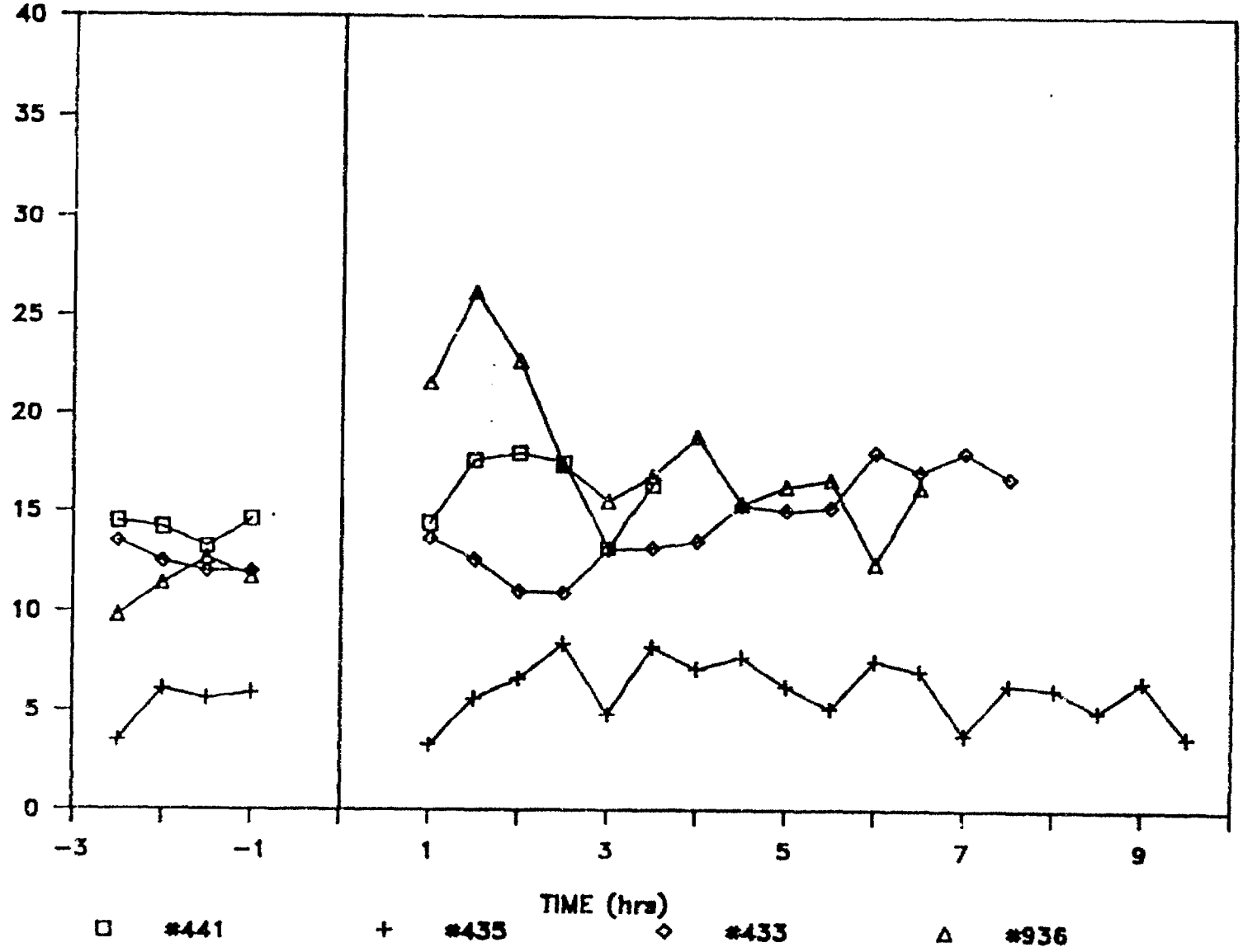



TFD
PULMONARY ARTERY PRESSURE
PA (mm Hg)
40
35
30
25
20
15
10
5
0
-3
-1
1
3
5
7
9
TIME (hrs)
□ #441
+ #435
◇ #433
△ #936


TFD
LEFT ATRIAL PRESSURE
LA (mm Hg)
30
25
20
15
10
5
0
-5
-3
-1
1
3
5
7
9
TIME (hrs)
□ #441
+ #435
◇ #433
△ #938

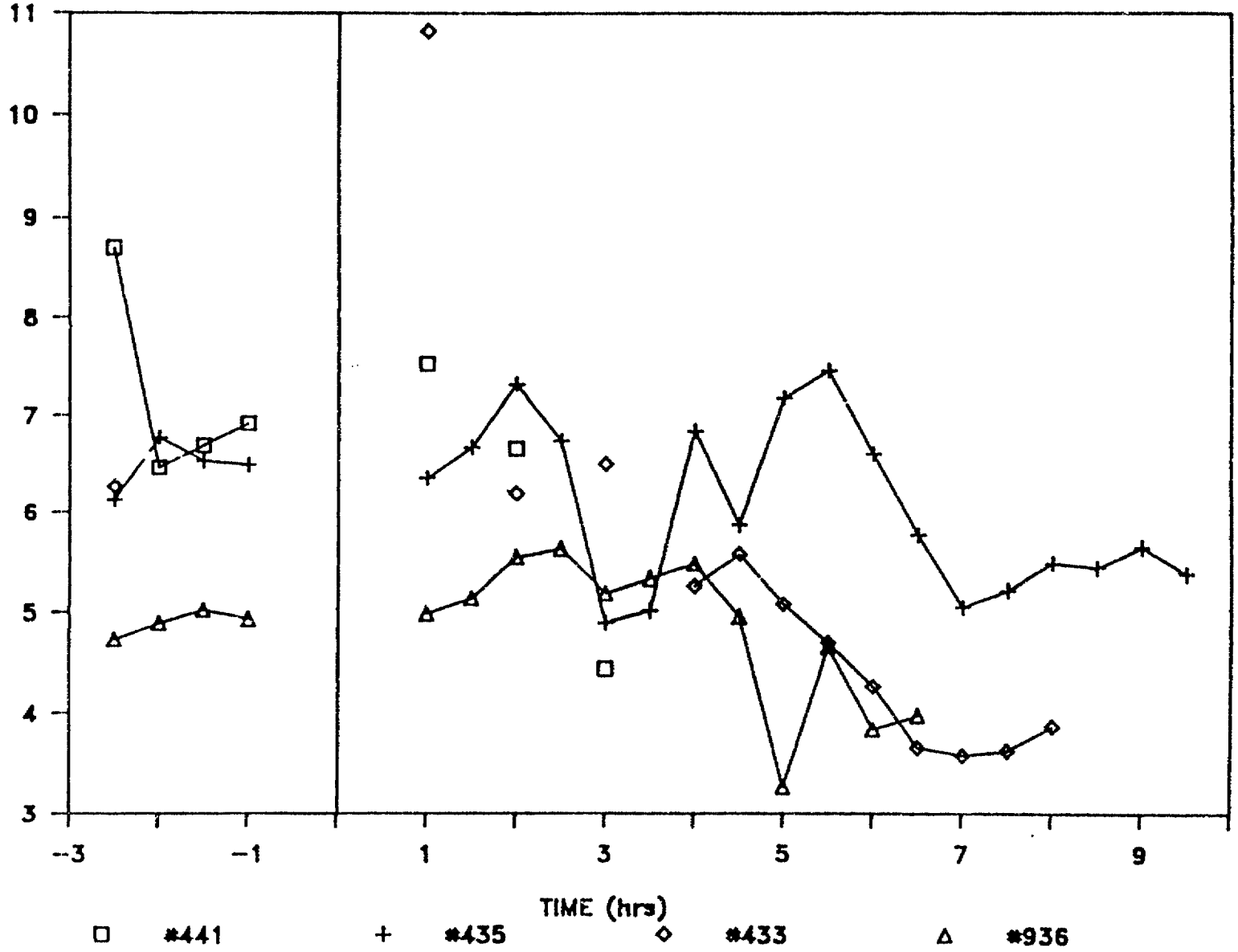
TFD

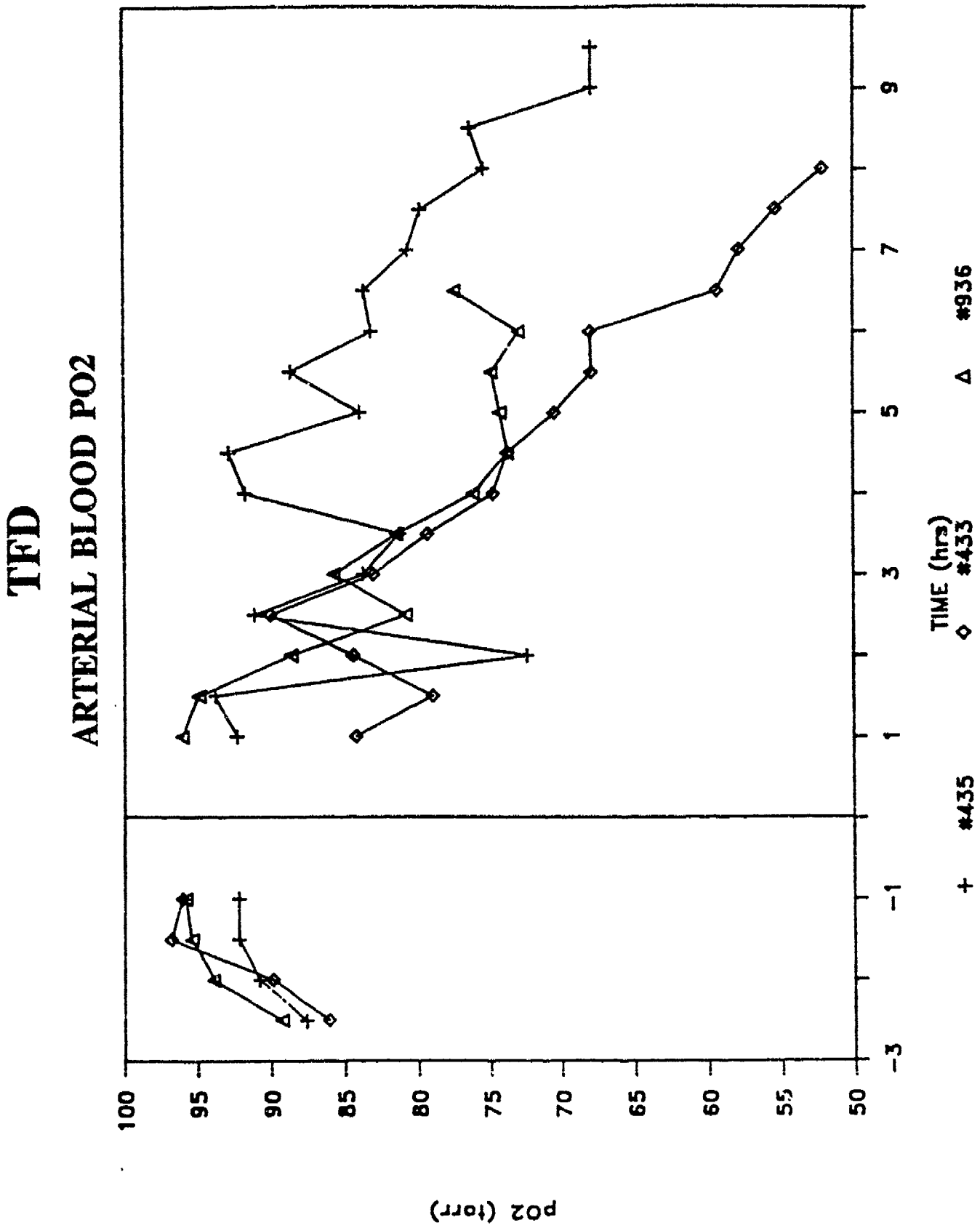
PULMONARY MICROVASCULAR PRESSURE

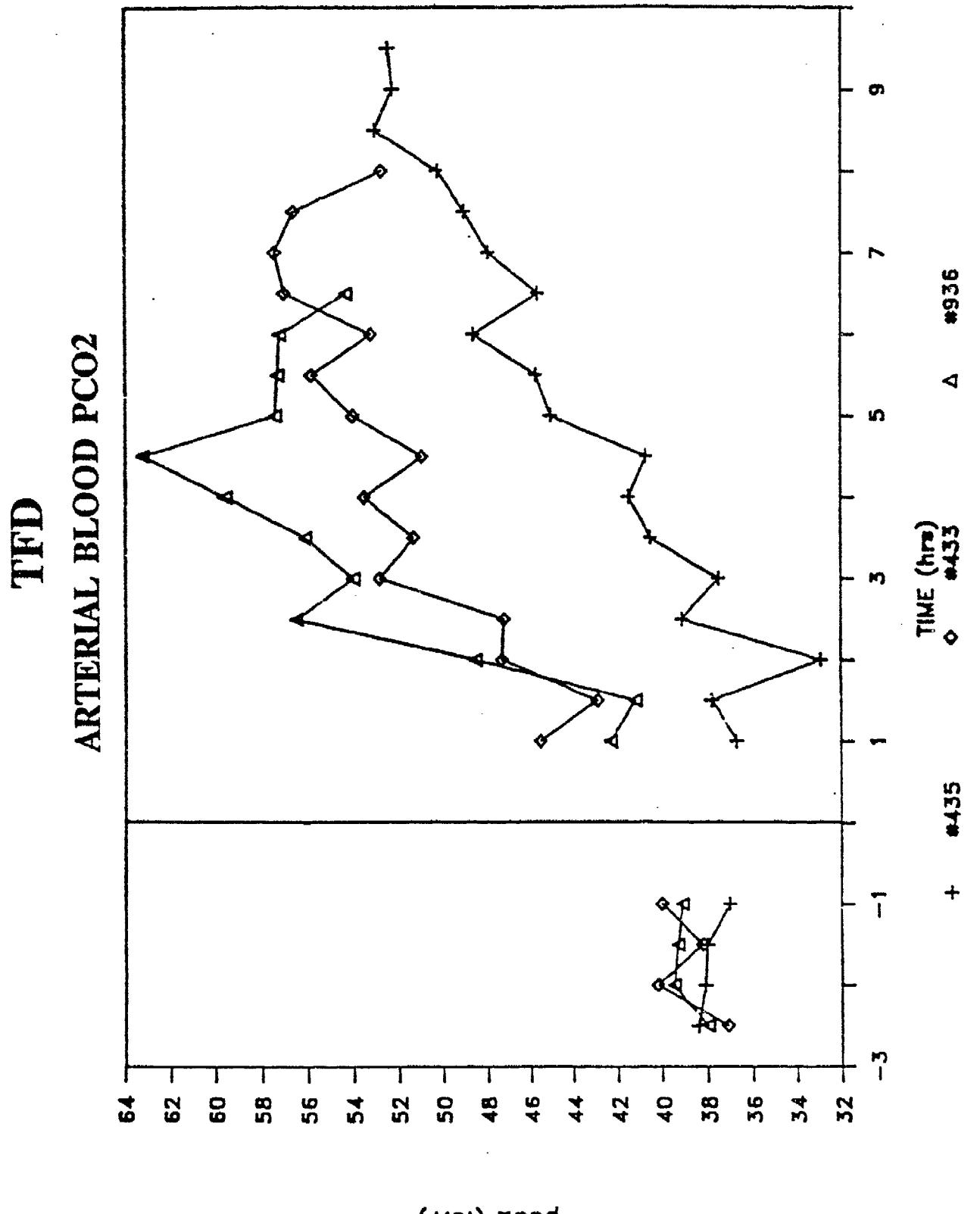


TFD

CARDIAC OUTPUT

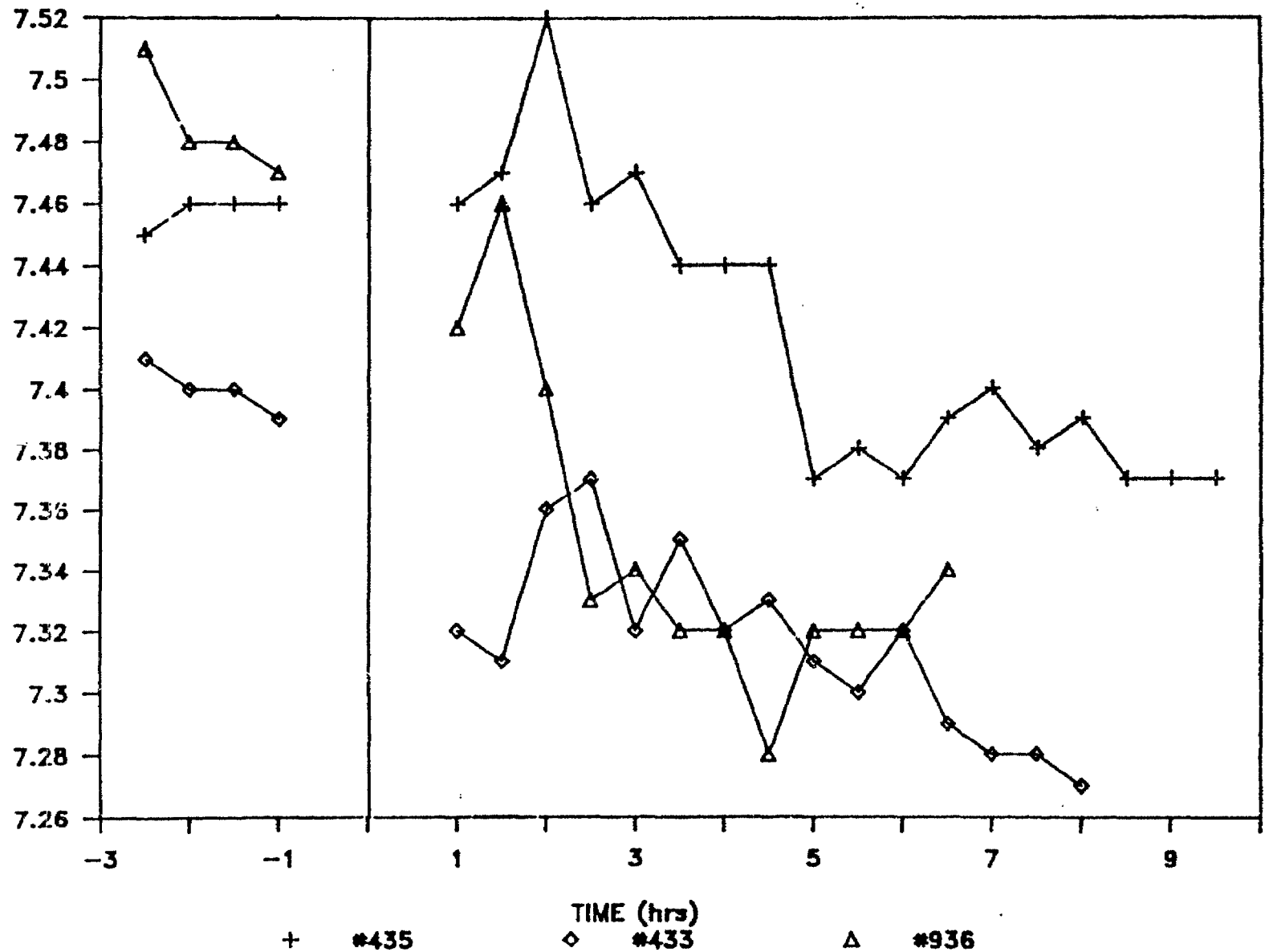



TFD
ARTERIAL BLOOD PO2
pO2 (torr)
100
95
90
85
80
75
70
65
60
55
50
-3
-1
1
3
5
7
9
TIME (hrs)
+ #435
◇ #433
Δ #936


TFD
ARTERIAL BLOOD PCO2
pCO2 (torr)
64
62
60
58
56
54
52
50
48
46
44
42
40
38
36
34
32
-3
-1
1
3
5
7
9
TIME (hrs)
+ #435
◇ #433
Δ #936

TFD

ARTERIAL BLOOD pH

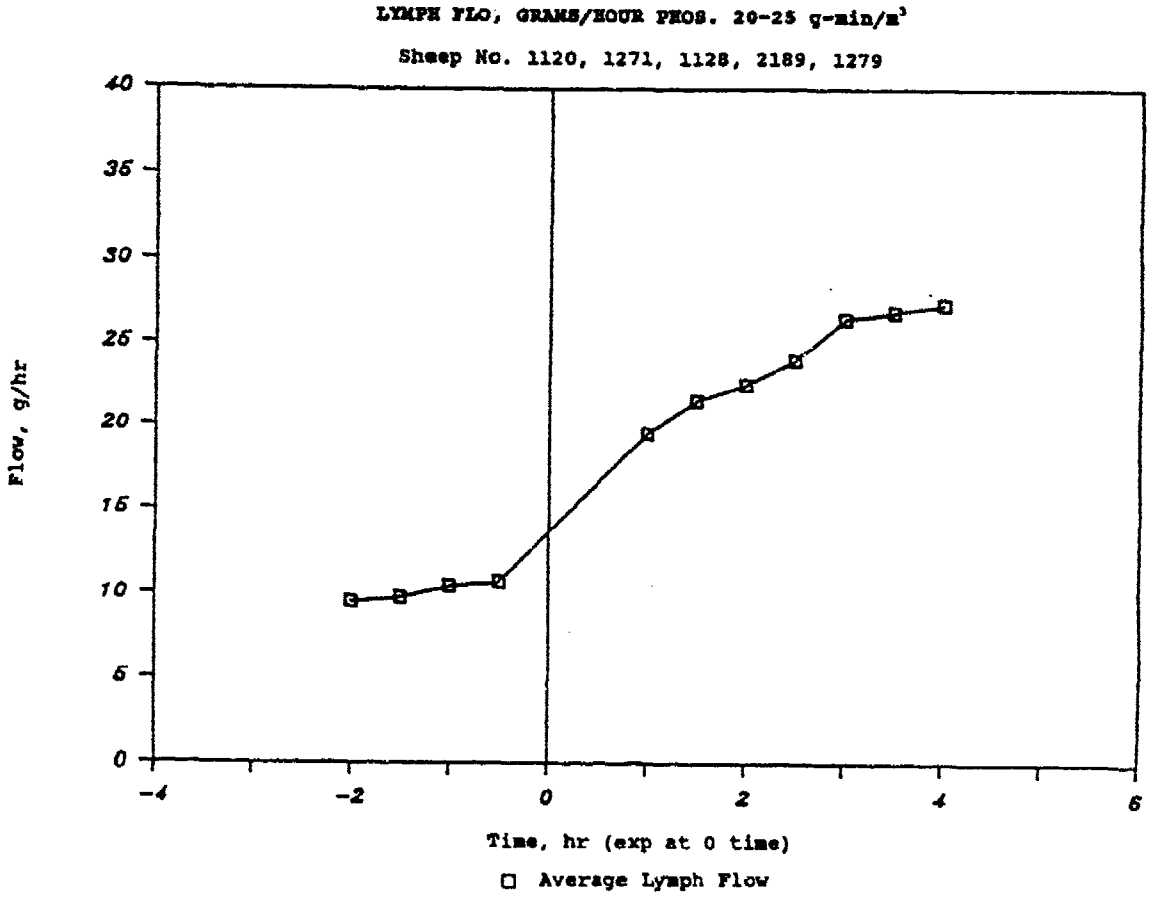


SUMMARY OF FINDINGS

- Both compounds produce a permeability-type pulmonary edema with delayed onset
- Possible mechanistic differences between PFIB and TFD
- Difficult to compare doses
- Lymph flow predicts edema long before signs and symptoms in sheep
- Sheep lung lymph model has utility for the study of interventions

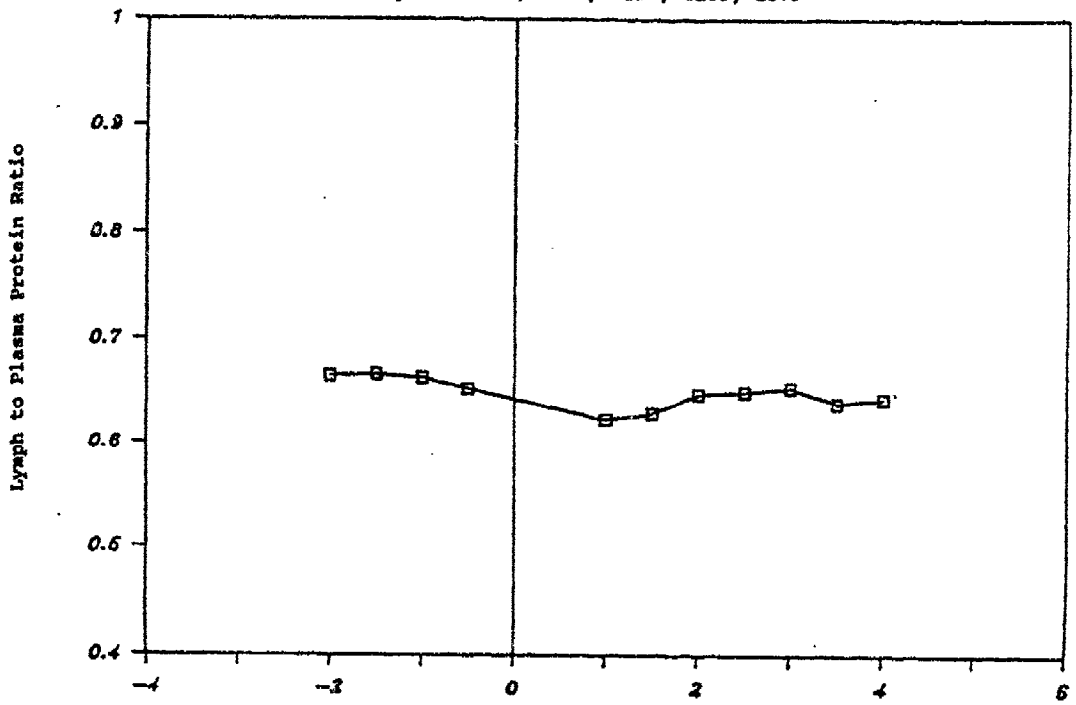
EFFECTS OF PHOSGENE IN A CHRONICALLY INSTRUMENTED SHEEP MODEL

(Presented by LTC Jill R. Keeler)



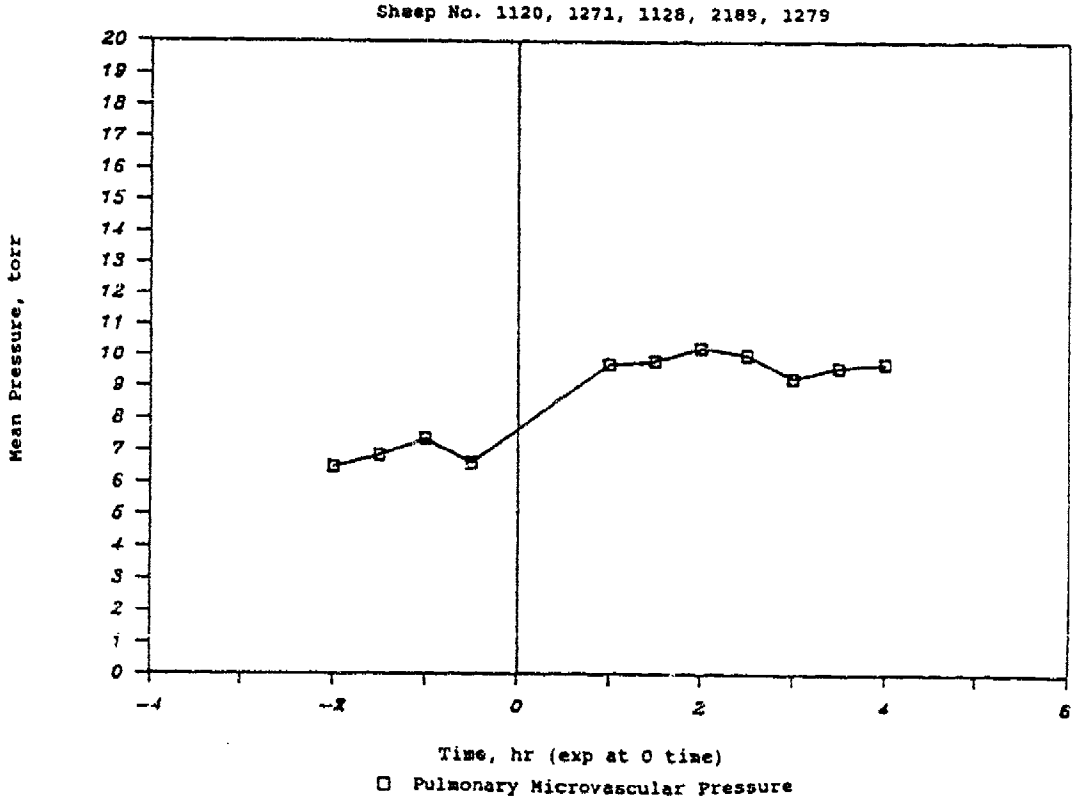
LYMPH TO PLASMA PROTEIN RATIO

Sheep No. 1120, 1271, 1128, 2189, 1279



MEAN PULMONARY MICROVASCULAR PRESSURE

Sheep No. 1120, 1271, 1128, 2189, 1279



DISCUSSION AFTER LTC JAEGER AND LTC KEELER'S PRESENTATION

Initial comments clarified that it was the pulmonary atrial pressure (PAP) that increased in conjunction with pulmonary microvascular pressure. It was also noted that PAP and left atrial pressure (LAP) were derived values. That is, they were not measured directly.

LTC Keeler clarified that the dose of phosgene administered to the sheep was 20-25 g/m^3 over a 10-minute period. A short discussion followed regarding the LCt_{50} of phosgene for sheep and humans. LTC Keeler noted that they had been surprised to find that sheep had apparently never been exposed to phosgene under similar experimental conditions. It was estimated that the LCt_{50} was equal to 13 g phosgene/m^3 for a 10-minute exposure where the observation point was 24 hours after exposure. Dr. Urbanetti commented that there was a moderate amount of human data available on accidental phosgene exposures, and he suggested that the LCt_{50} for sheep was approximately two times that for humans. He added that, in humans, as the dose increases there is a tendency to get more small-airway dysfunction. LTC Jaeger responded by noting that the dose-response curve for PFIB was steep, and with PFIB, the airways were filled with a frothy white fluid, whereas with TFD and phosgene, there was a large increase in the amount of mucus accumulation in the airways. Finally, it was reemphasized that, in order to establish the precise dose of inhalant administered, it would be necessary to perform experiments with anesthetized sheep which were artificially ventilated.

Dr. Said noted that any increase in microvascular pressure will exacerbate the edema. However, it's not clear whether the increased microvascular pressure was due to heart failure or if it was a pulmonary problem. Was the microvascular pressure increase caused by vascular constriction with no increase in LAP, or was there an increase in LAP indicating left heart failure? To this, LTC Jaeger responded that there was no evidence of left heart failure. LTC Keeler added that the measurement of left atrial pressure (LAP) was not that reliable, and that they had relied more upon the PAP value. Dr. Fowler noted that it was interesting both that the onset of hypoxemia was so rapid and that it was not associated with a spike in PAP. LTC Jaeger, however, did not think that the magnitude of the decrease in PAP was all that dramatic until the end of the experiments.

The discussion then focused on the relative importance of interstitial edema versus interalveolar edema, with LTC Jaeger speculating that interstitial edema is not as great a threat as the interalveolar edema, which is usually fatal. Dr. Mark

cautioned about jumping to this conclusion, indicating that other things may be responsible for death from chronic lung disease, using the example of collagen layers lining the alveoli and thus interfering with gas exchange.

PILOT STUDY TO EVALUATE ANALYTICAL METHODS FOR DETECTION OF TFD AND TO DETERMINE THE EFFECTS OF INTRAPERITONEAL INJECTION IN RATS

LTC Jill R. Keeler

The primary objective of this project was to determine if TFD or a metabolite of TFD, after its i.p. injection into rats, could be detected in blood and in expired gases. Analytical methods included FTIR (Fourier transform infrared) spectrometry and gas chromatography (GC) head-space analysis. FTIR was chosen for its ability to quantitate the concentration of components of a gaseous mixture and to identify specific metabolites. The study was designed so that the nature of the respiratory effluent could be assessed and so that histopathologic changes could be determined. The i.p. doses of neat, liquid TFD (0.5, 5, and 50 μl) were about five-, fifty-, and five-hundredfold greater than the median lethal dose for inhalation exposure. Rats were sacrificed by decapitation 30 minutes after injection.

Results indicated no evidence of volatile parent compound or metabolites in blood. Further, there was no evidence that TFD or any recognizable metabolite was eliminated by the lungs. Analysis (by GC) of water, sheep plasma, and sheep RBC samples spiked with TFD revealed that TFD was stable for several hours in water, but was rapidly lost from plasma and RBCs. The half-life of TFD in plasma was about 85 minutes and in RBCs about 25 minutes. The short half-life may explain why TFD could not be easily detected in rat plasma after injection; i.e., TFD may indeed have entered the blood after i.p. injection but was "lost" before the sample could be analyzed. Finally, it was notable that rats died after the highest dose of TFD but not from pulmonary edema; in fact, no substantial histopathology of any organ could be detected at autopsy.

PILOT STUDY TO EVALUATE ANALYTICAL METHODS FOR DETECTION OF TFD AND TO DETERMINE THE EFFECTS OF INTRAPERITONEAL INJECTION IN RATS

MAJ Kenneth G. Phillips, MAJ David H. Moore,
Dr. Ming L. Shih, Dr. Ernest H. Braue,
and LTC James B. Nold

(Presented by LTC Jill R. Keeler)

U.S. Army Medical Research Institute of Chemical Defense
Aberdeen Proving Ground, Maryland

THE EFFECT OF INTRAPERITONEAL INJECTION OF TFD ON RESPIRATORY EFFLUENT, BLOOD, AND PATHOLOGY OF RATS

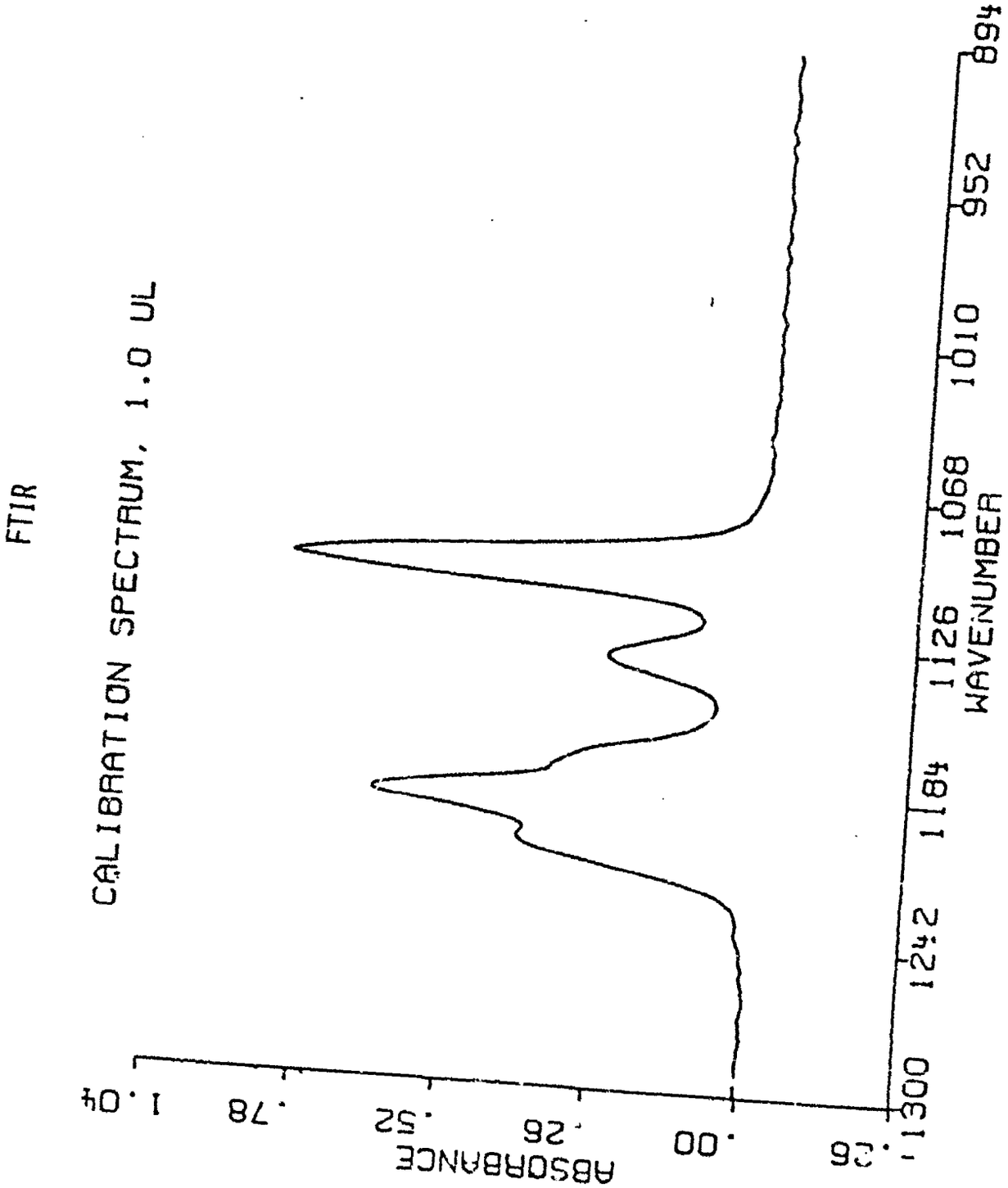
QUESTIONS

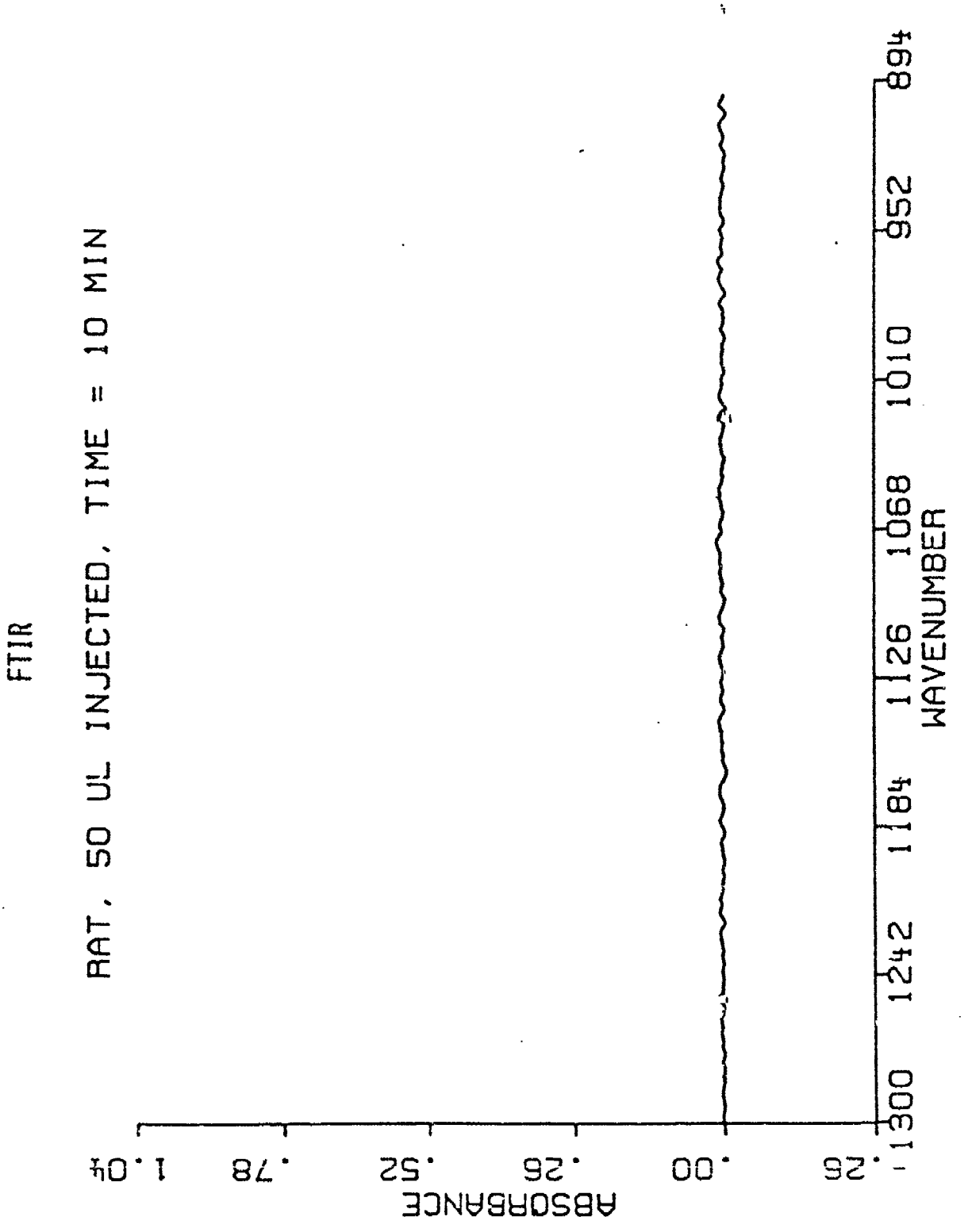
- Can this compound or a metabolic byproduct be detected in blood after i.p. injection?
- What was the nature of the respiratory effluent after an i.p. injection?
- Do the animals die after an i.p. injection?
- What is the nature of any observed morbidity or mortality?
- What histopathologic changes occur after i.p. injection?

Analysis System

Total volume of system is about 8 liters

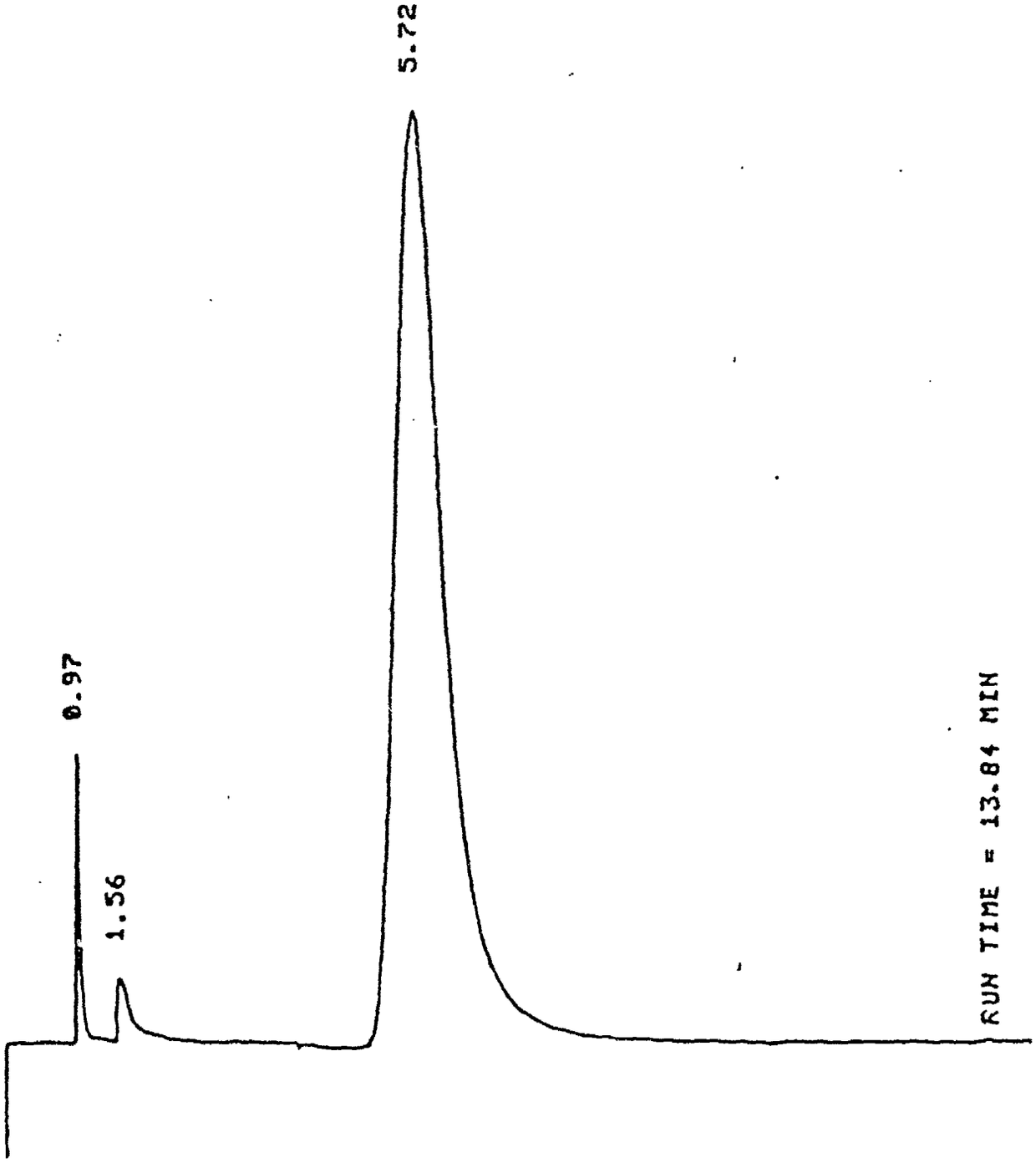
A = Small animal exposure apparatus
B = Air circulation pump
C = Miran IR Analyzer
D = FT-IR 10m gas cell


FTIR
CALIBRATION SPECTRUM, 1.0 UL
ABSORBANCE
-.26
.00
.26
.52
.78
1.04
1300
1242
1184
1126
1068
1010
952
894
WAVENUMBER

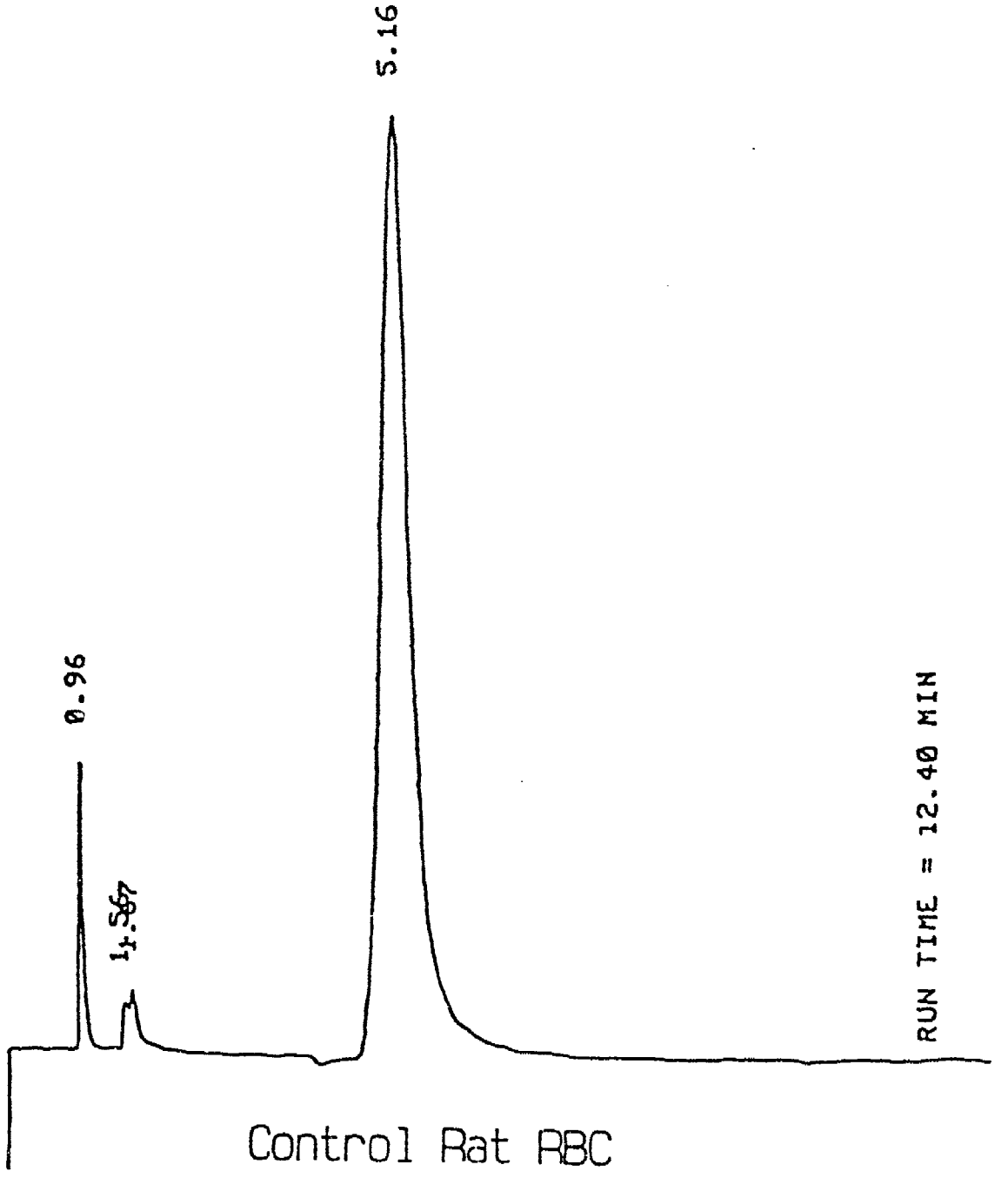

FTIR
RAT, 50 UL INJECTED, TIME = 10 MIN
ABSORBANCE
1.0+
.78
.52
.26
.00
-.26
WAVENUMBER
1300
1242
1184
1126
1068
1010
952
894

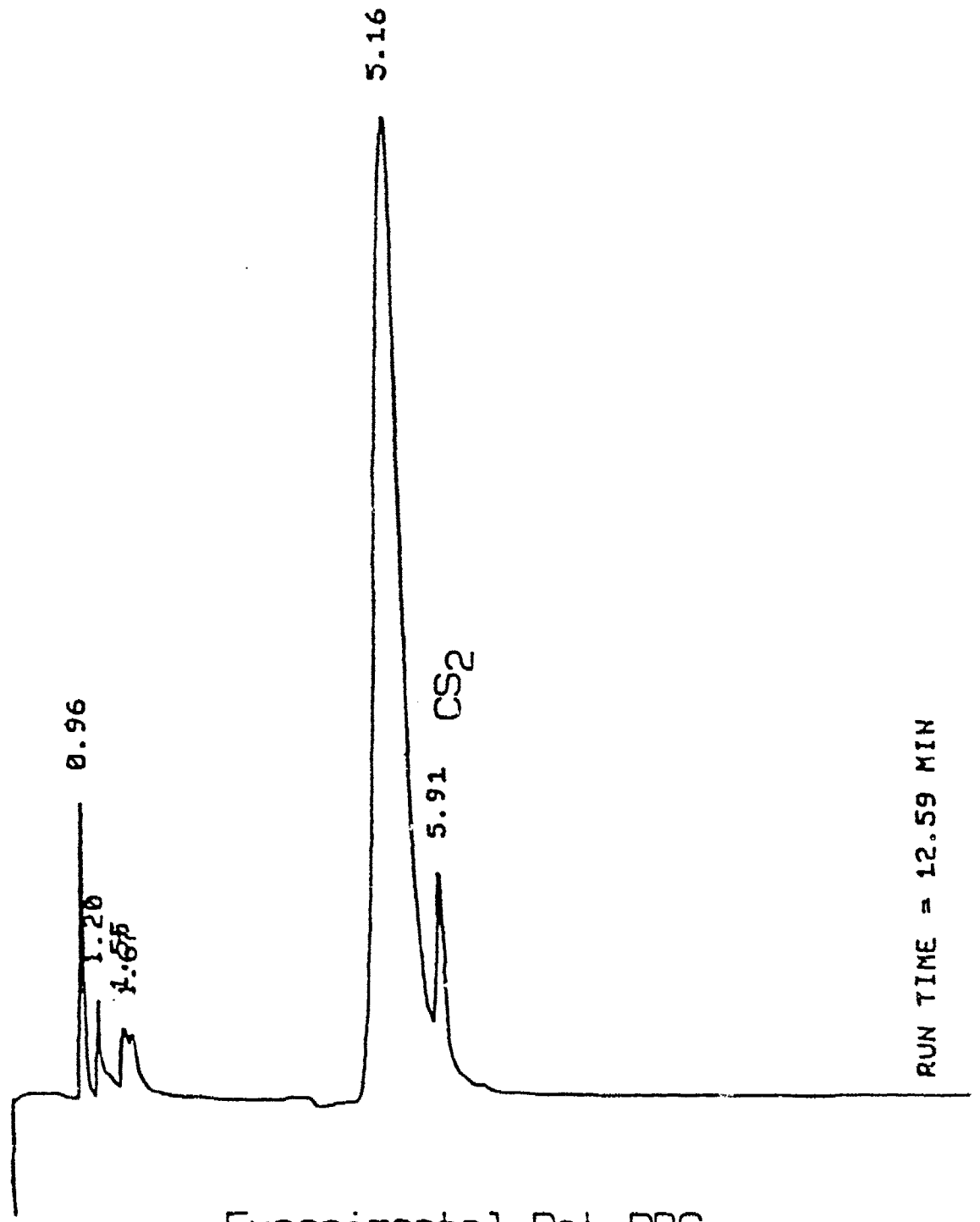
RUN TIME = 13.40 MIN
Room Air Injection (5 ul)
1.55
0.97

RUN TIME = 13.55 MIN
5.74
1.56
0.96
TFD Standard in Air [9 x 10(-5) mg/ml]

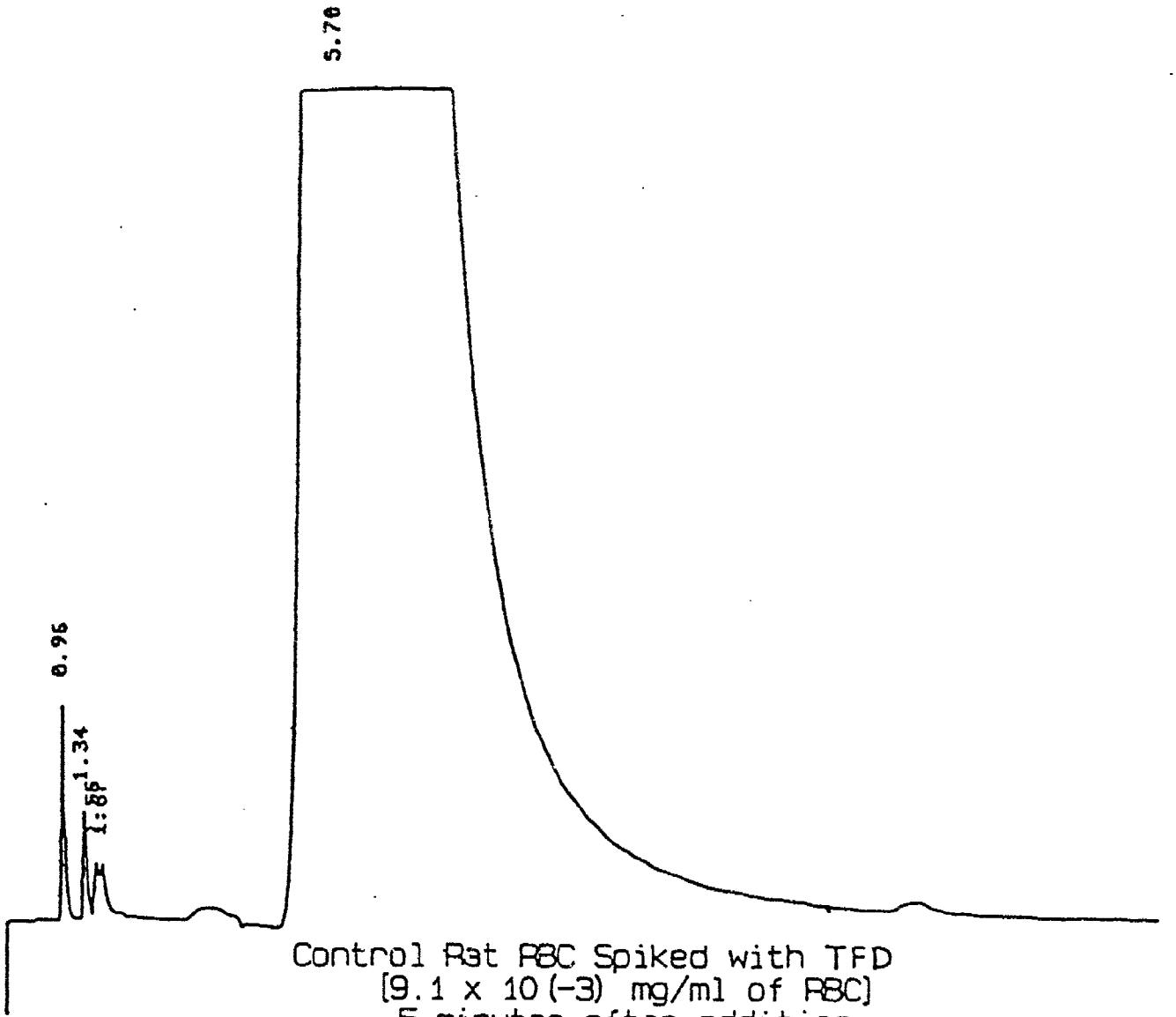


TFD Standard in Air [2.3 x 10(-4) mg/ml]

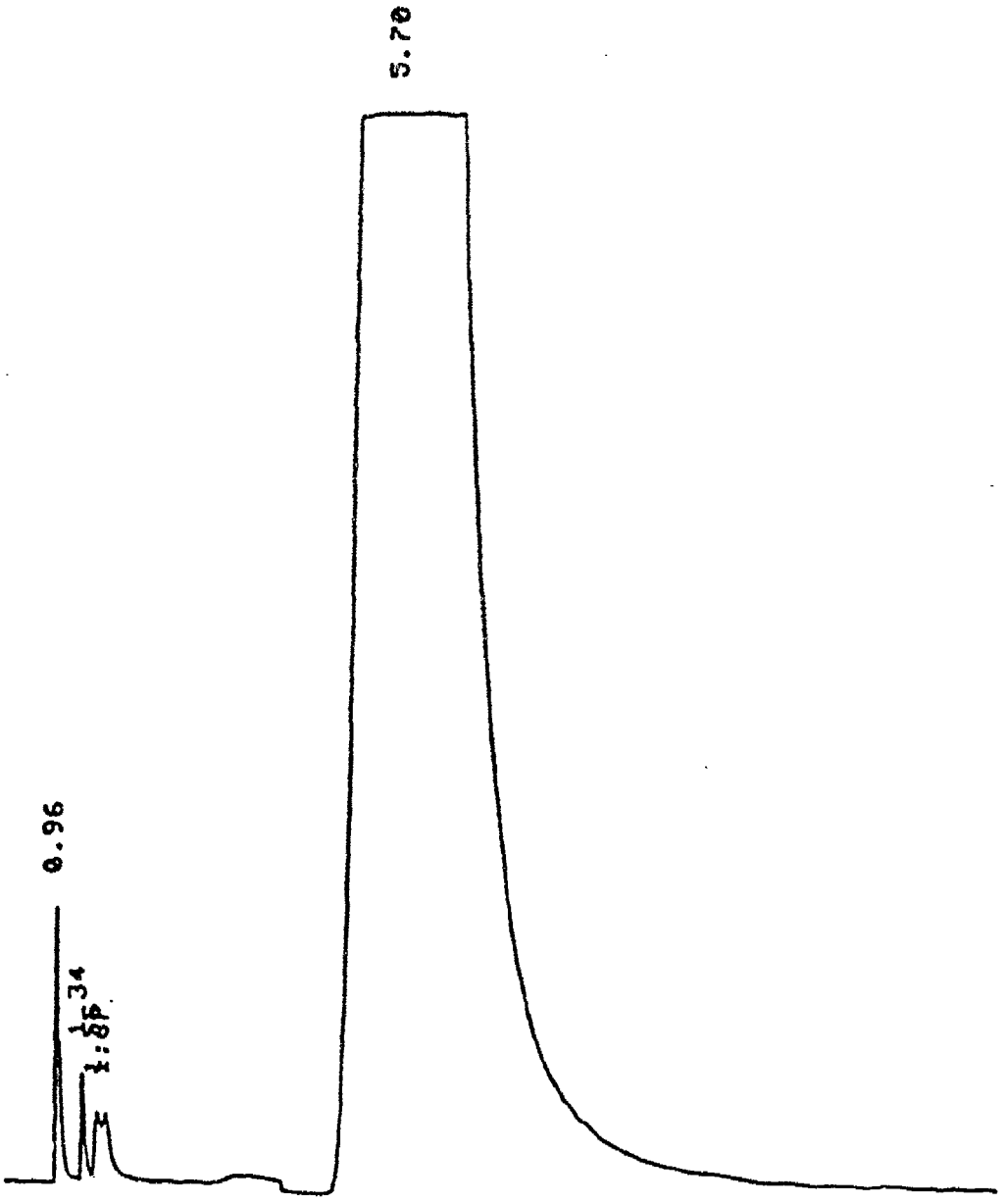

5.16
0.96
1.567
RUN TIME = 12.40 MIN
Control Rat RBC



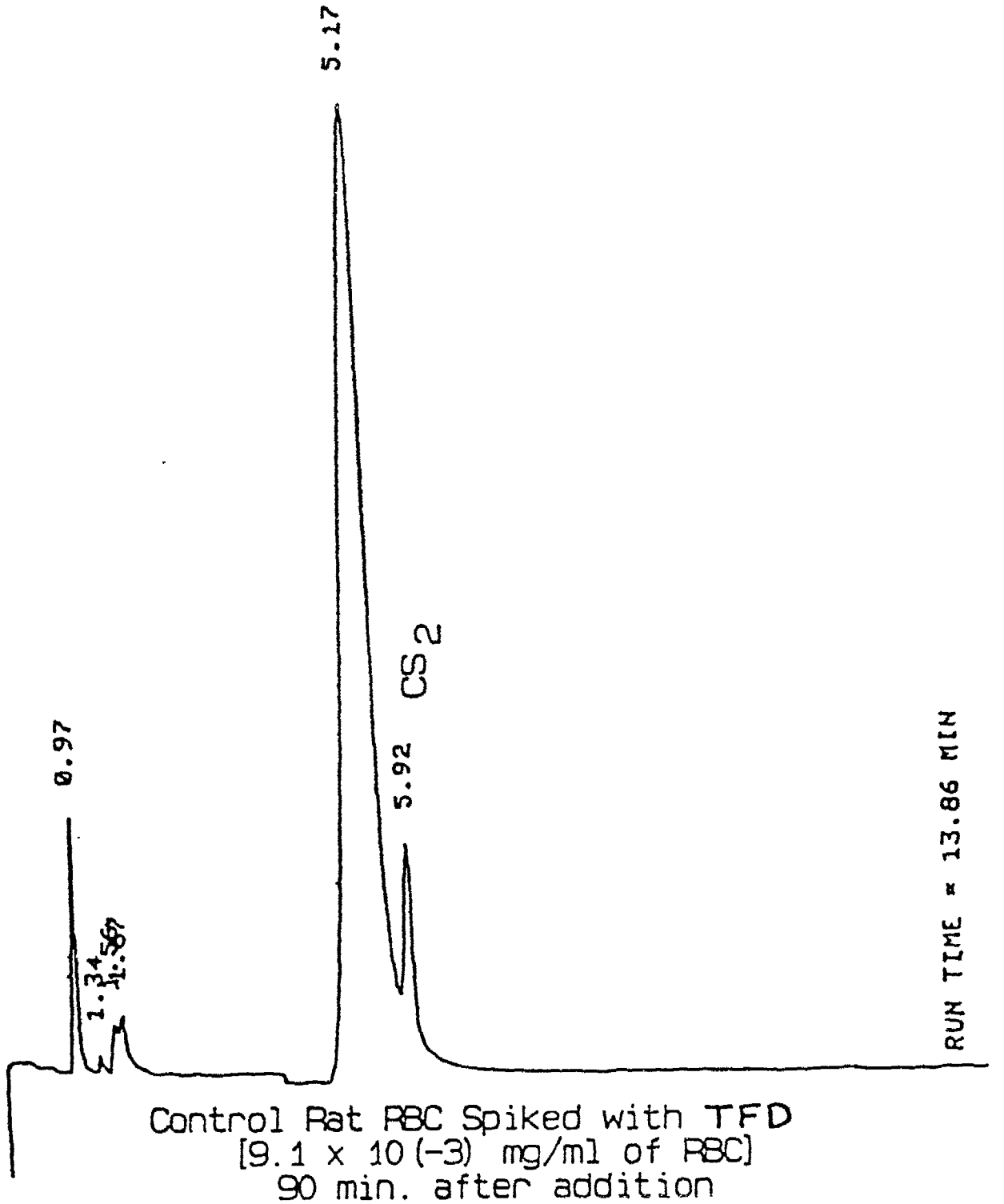
Experimental Rat RBC



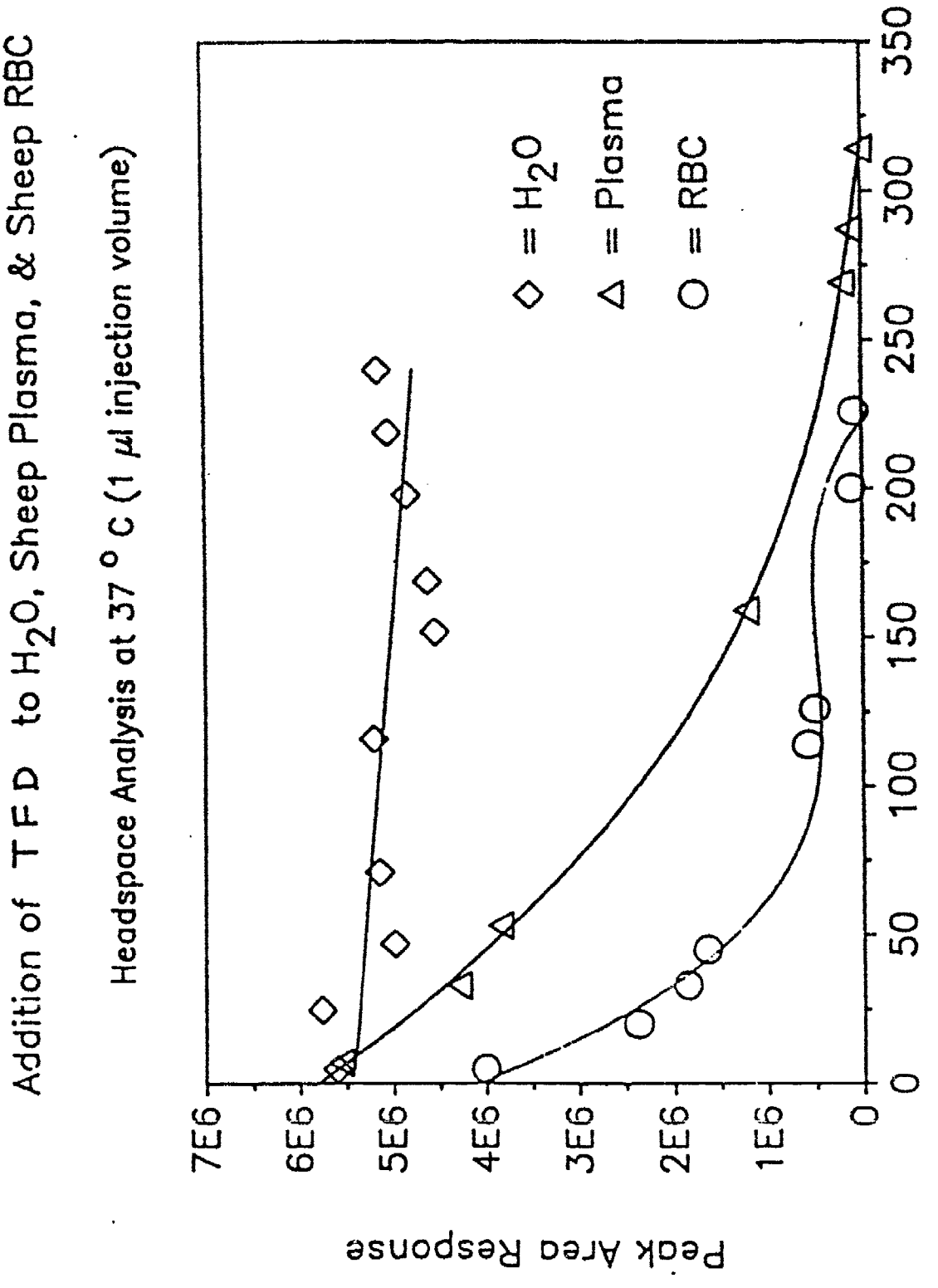
Control Rat RBC Spiked with TFD
[9.1 x 10(-3) mg/ml of RBC]
5 minutes after addition



Control Rat RBC Spiked with TFD
[9.1 x 10(-3) mg/ml of RBC]
45 min. after addition



Control Rat RBC Spiked with TFD
[9.1 x 10 (-3) mg/ml of RBC]
90 min. after addition


Addition of TFD to H_2O, Sheep Plasma, & Sheep RBC
Headspace Analysis at 37 °C (1 µl injection volume)
◇ = H_2O
△ = Plasma
○ = RBC
Peak Area Response
7E6
6E6
5E6
4E6
3E6
2E6
1E6
0
0
50
100
150
200
250
300
350
Minutes After Addition of TFD
(Spiked Concentration 75.6 µg/cc)

CONCLUSIONS OF I.P. RAT STUDIES

- Analytical methods were developed.
- No detectable volatile parent compound of metabolites are present in blood.
- No evidence that parent compound or any recognizable volatile metabolites are eliminated by the lungs.
- Rats did not die of pulmonary edema.

DISCUSSION AFTER LTC KEELER'S PRESENTATION

In response to a question from Dr. Lehnert, LTC Keeler indicated that specific efforts had not been made toward determining if there was an effect of TFD on the oxygen-carrying capacity of the red blood cells. Dr. Shih added that upon casual inspection, there were no visible morphological changes in the red blood cells. Dr. Lehnert then commented that such measurements might be useful in determining possible effects of TFD on red blood cell metabolism.

The discussion then was directed to the metabolism of TFD. LTC Keeler indicated that, at this point, not much about this topic was established. She said that CS_2 and a carbamyl sulfide were found in the blood; it was not known, however, if they were important metabolites of TFD. Clearly, access to radiolabeled TFD would be quite useful. LTC Keeler indicated that attempts were under way to develop a radioimmunoassay, with progress having been made toward producing an antibody [for TFD]. MAJ Ripple asked why they just couldn't measure fluorine. LTC Keeler said that the electron capture device does indeed pick up halogens, sulfur, and oxygen in metabolites.

Dr. Mark noted that in early pulmonary edema the protein content of edema fluid is very low and doesn't stain well; therefore, successful light microscopy is unlikely. Further, it is usually difficult to differentiate between interstitial and alveolar edema at the early stages. One way to get around this, he added, was the simple measurement of lung wet weights which, although seemingly a crude method, was very effective in determining if edema was or was not present. LTC Keeler responded that they had not measured wet weights of lungs from animals in the experiments she had described.

Mr. Harris raised the question of why the drug was administered i.p. instead of i.v., and of what influence that method would have on determining the metabolism of the drug. Dr. Hurt indicated that he thought that if the compound was injected i.v. it might turn into a gas and then a pulmonary embolism might result. LTC Keeler responded that Dr. Assaad would soon be doing a study designed to evaluate different routes of exposure.

COL Dunn summed up this discussion by saying that the compound (TFD) was injected and, obviously, it went somewhere. He stated that the people at ICD were currently trying to determine where it went and how it was metabolized and suggested that the forthcoming radiolabeled compound would help them to meet that goal.

OVINE ENDOTHELIAL CELLS FOR THE STUDY OF PERMEABILITY AND MECHANISMS REGULATING PULMONARY EDEMA

Dr. Robert J. Werrlein

These research efforts have served to establish primary cultures of pulmonary endothelial cells from the proximal and distal passages of rat and sheep airway epithelium. Pure lines of seven different cell types have been isolated and conditions for reproducible growth in culture have been optimized. This has allowed the study of cell morphology and physiology, and the development of experimental systems for evaluating membrane permeabilty using microcarrier beads. In other work, populations of rat tracheal epithelial cells were induced to undergo reversible differentiation to an astroglia-type cell, which has subsequently been isolated and purified.

Methods for verifying cell types and optimizing growing conditions were described. It is now possible to cryopreserve and store pure cell lines for future experimentation, resulting in the accumulation of a valuable research asset at USAMRICD.

The experimental system for investigating the direct and indirect effects of agent-induced cytopathology was discussed. Results to date indicate that airway epithelium produces low-molecular-weight mediators, and data suggest that permeability is affected by these mediators. It has also been found that neuroendocrine cells are sensitive to the composition of inhaled air, and that they secrete intrapulmonary mediators. Finally, it was observed that relaxation of vascular smooth muscle potentiates fluid efflux from the walls of blood vessels, and that a moderate increase in perivascular potassium has a distinct effect on vascular smooth muscle tension.

OVINE ENDOTHELIAL CELLS FOR THE STUDY OF PERMEABILITY AND MECHANISMS REGULATING PULMONARY EDEMA

Dr. Robert J. Werrlein, Janna S. Madren,
and Alan D. Knapton

U.S. Army Medical Research Institute of Chemical Defense
Pathophysiology Division
Aberdeen Proving Ground, Maryland

RESEARCH OBJECTIVES

- Establish primary cultures of pulmonary endothelial cells
- Isolate a pure cell line
- Optimize conditions for growth in culture
- Cryopreserve and store pure lines for future experiments
- Determine "normal" morphology and physiology of cells
- Develop experimental systems for permeability studies
- Expose cells to threat agents and determine effects

VERIFICATION OF CELL TYPE

T.E.M

Weibel-Palade Bodies

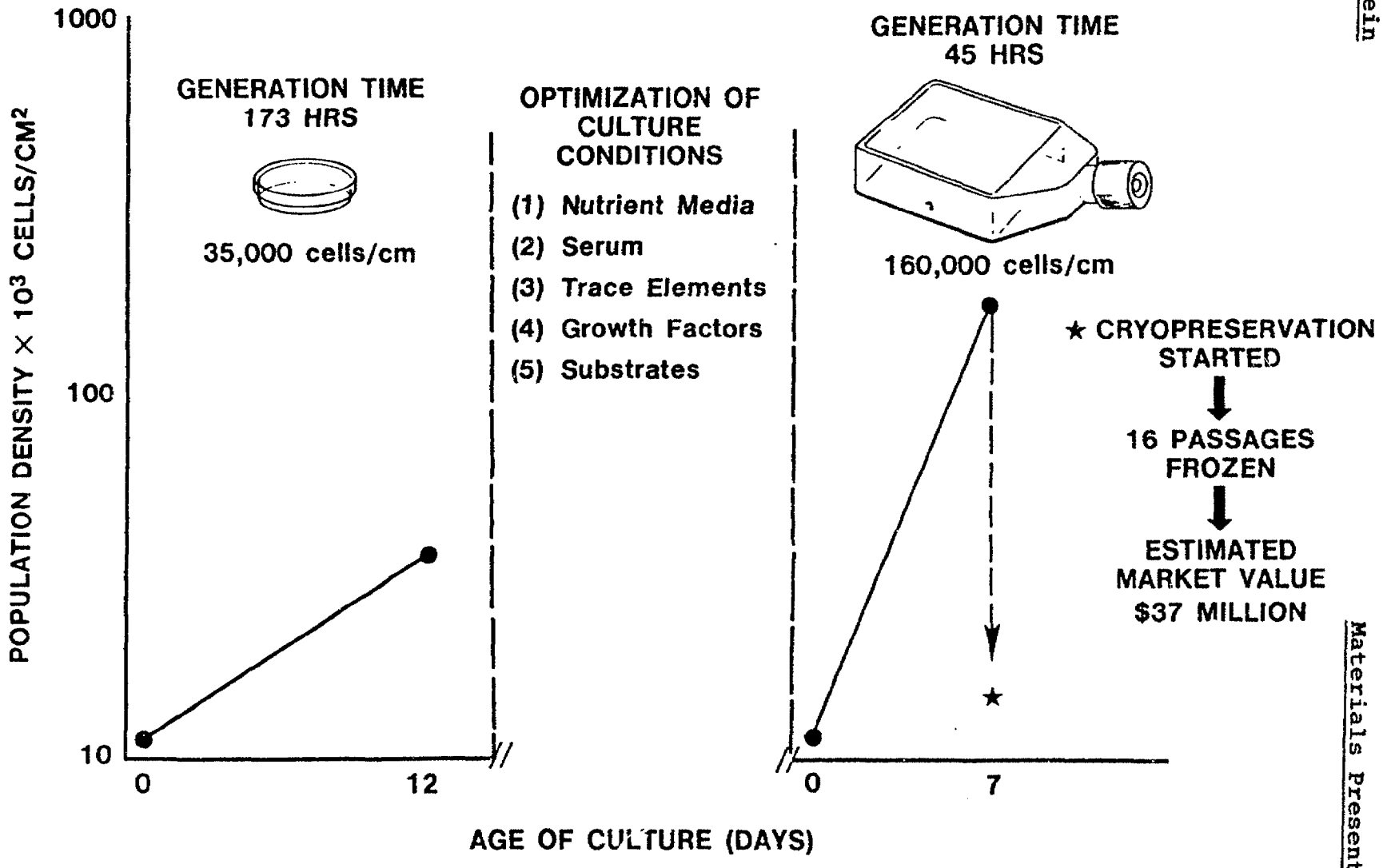
Immunohistochemistry

Test for Von Willebrand Factor

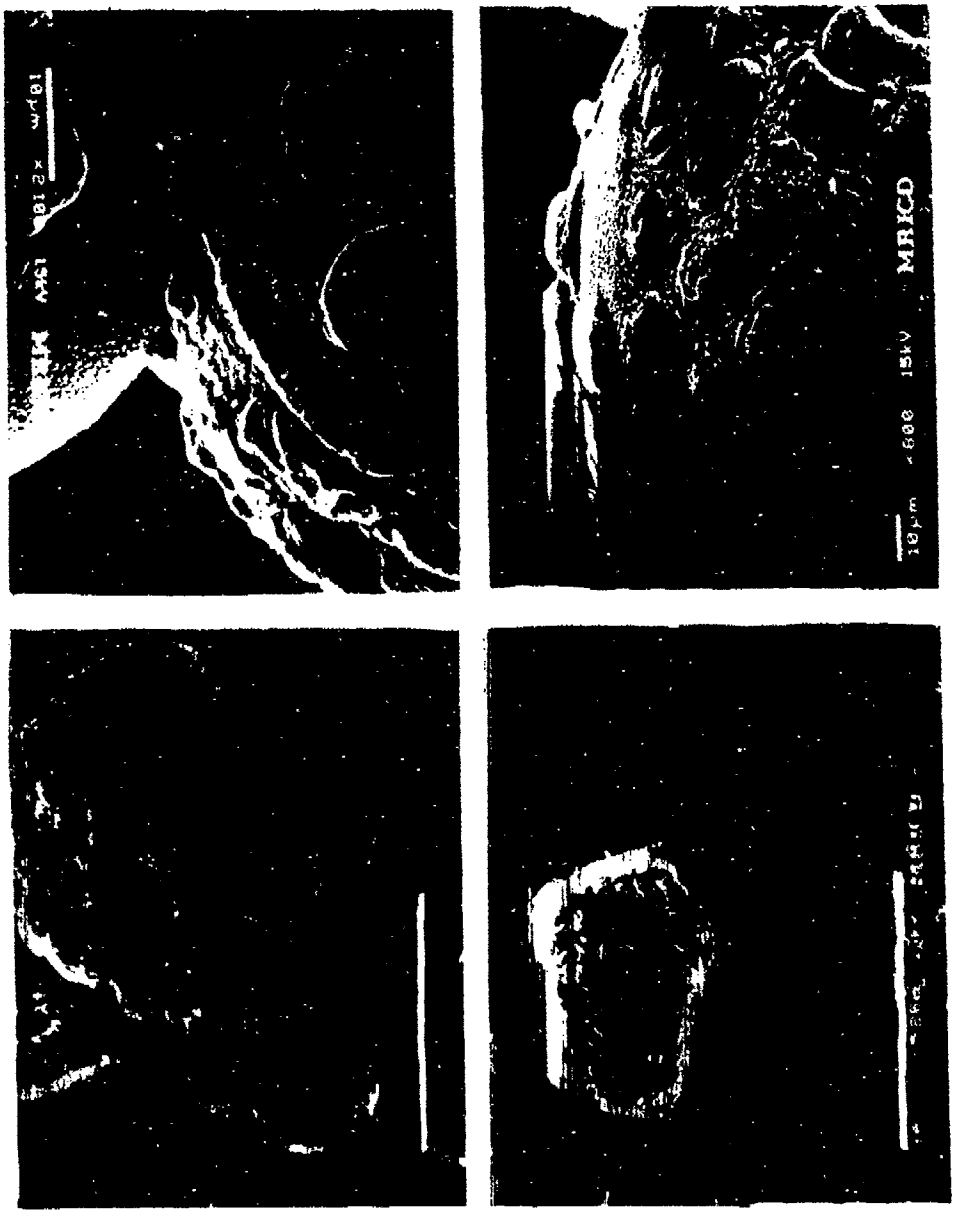
1) r x h F-VIII

2) s x r Ig -FITC

OPTIMIZATION OF GROWTH

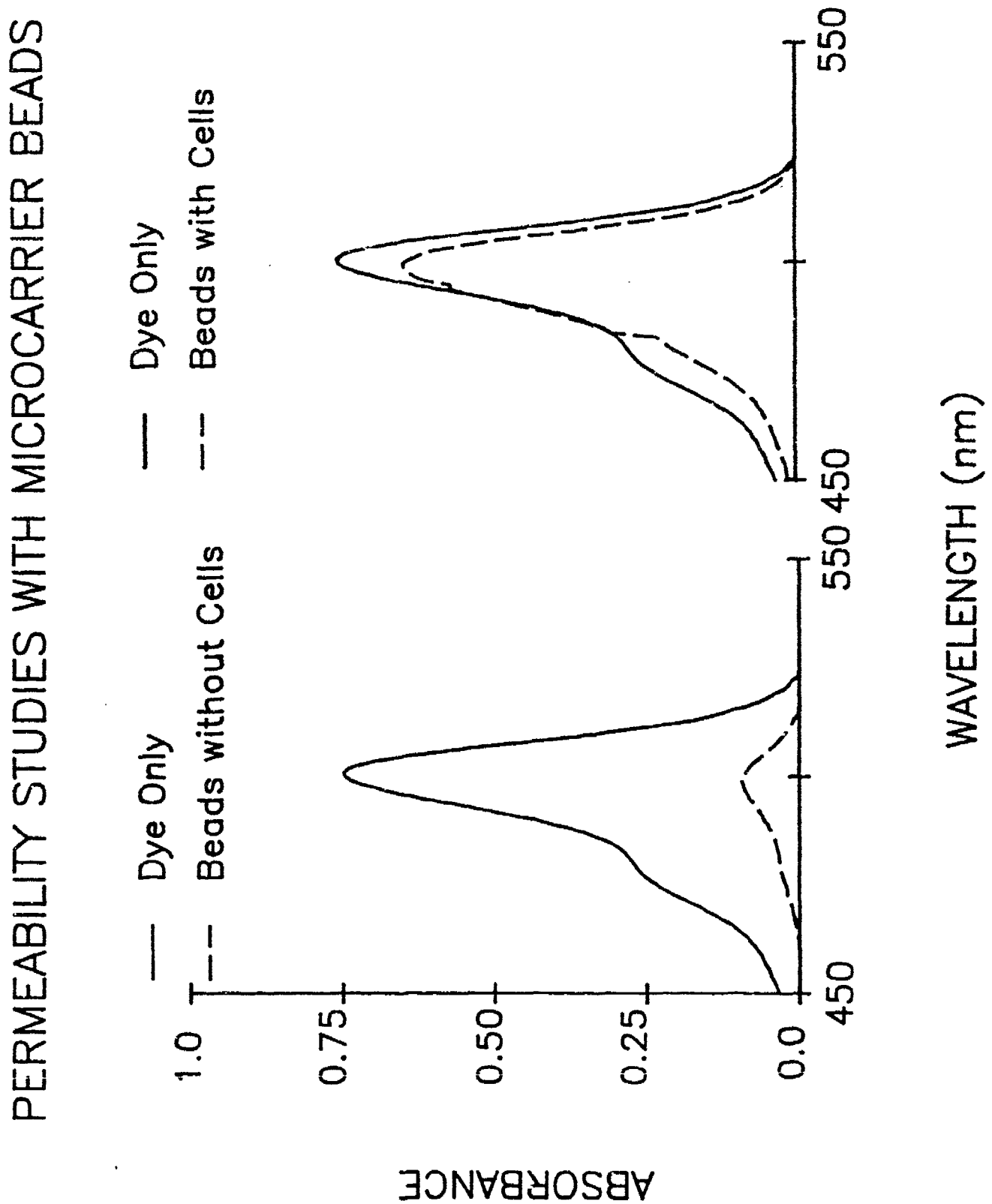


CELL TO SUBSTRATE ATTACHMENTS



CELL TO CELL ATTACHMENTS

MICROCARRIER CULTURES FOR PERMEABILITY STUDIES


PERMEABILITY STUDIES WITH MICROCARRIER BEADS
Dye Only
Beads with Cells
Dye Only
Beads without Cells
1.0
0.75
0.50
0.25
0.0
ABSORBANCE
450
550
450
550
WAVELENGTH (nm)

EVIDENCE FOR INDIRECT AGENT EFFECTS

- Permeability is affected by low-molecular-weight mediators
- Airway epithelium produces low-molecular-weight mediators
- Tracheal explants produce astroglial-type cells
- Neuroendocrine cells are sensitive to the composition of inhaled air, and secrete intrapulmonary mediators
- Relaxation of vascular smooth muscle potentiates fluid efflux from blood vessel walls
- Moderate increase in perivascular K^+ activity (up to 10 mM) has a distinct effect on vascular smooth muscle tension

SUMMARY

- Seven different cell types have been isolated from the proximal and distal passages of rat and sheep airway epithelium
- We have developed nutrient media and substrates which support the reproducible growth of these cells in culture
- We have induced a population of rat tracheal epithelial cells to undergo reversible differentiation to an astroglial-type cell
- We have isolated and purified this cell line
- We have produced sophisticated experimental systems for investigating the direct and indirect effects of agent-induced cytopathology

DISCUSSION AFTER DR. WERRLEIN'S PRESENTATION

Two aspects of methodology were discussed. First, Dr. Werrlein indicated that the erythrosin B method was easier to use in his studies of endothelial cell monolayer permeability than other dye exclusion methods, such as trypan blue. Nigrosin dye exclusion is also good, but doesn't work well when spectrophotometry is needed.

Dr. Werrlein commented on the prospects of future studies with this cell system, noting that it will be possible to examine factors which are mediators, such as hyperpolarizing factor, constricting factor, and relaxation factor. All of these effects can be monitored in the suspension culture in which samples can be drawn out and analyzed. Also, it will be possible to evaluate parameters such as cellular respiration and PO_2 levels. The point was made by Dr. Lehnert that it may be a good idea to prepare cultures that contain macrophages since they are omnipresent in conducting airways and may be important mediators in various biochemical and physiologic processes. Dr. Werrlein indicated that they do separate those cells but that the cells die in culture after about 21 days, and that it may be necessary to replenish the cultures with macrophages. Finally, Dr. Werrlein indicated that they do not have a major problem regarding overgrowth with fibroblasts.

Dr. Mark noted that there appeared to be similarities between the ovine endothelial cells studied by Dr. Werrlein and references in the literature to meninges- and astroglia-like cells that are found in various pulmonary tumors. He added that various metastatic cancers apparently produce vasogenic substances. He thought that the model system presented by Dr. Werrlein could be used to study both growing cells and confluent cells and may prove to be a very useful system.

PHOSGENE: CLINICAL IMPORTANCE AND MANAGEMENT

Dr. John S. Urbanetti

The chemistry, pharmacology, and toxicology of phosgene was discussed in this presentation. Phosgene gas produces a delayed pulmonary edema (approximately 24 hours elapses between exposure and symptomology). Phosgene hydrolyzes in the moist respiratory tract to hydrochloric acid and carbon dioxide. A primary effect of phosgene is disruption of normal alveolar membranes and capillary membrane permeability. The gas is toxic primarily in the more peripheral airways. Acute respiratory effects range from coughing and lacrimation at low doses to severe cough with laryngospasm (possibly leading to sudden death) at higher doses. Animal studies reveal striking temporal histopathological effects of high doses of phosgene including bronchiolar constriction, swelling, necrosis, and perivascular edema as early as 30 minutes after exposure. (Urbanetti, J.S. [1988]. Battlefield chemical inhalation injury. In *Pathophysiology and Treatment of Inhalation Injuries*, J. Luke, ed. Marcel Dekker, New York.)

Four case histories of exposure to phosgene were discussed in detail, with emphasis on clinical signs and symptoms and treatment modalities.

PHOSGENE: CLINICAL IMPORTANCE AND MANAGEMENT

Dr. John S. Urbanetti

INTRODUCTION

Phosgene is a particularly important toxic agent for two clinical reasons. First, the substance itself is widely available and remarkably toxic to the lung; second, the physiologic abnormalities it produces are strikingly similar to adult respiratory distress syndrome (ARDS) in presentation, histopathology, and clinical response (or nonresponse) to treatment.

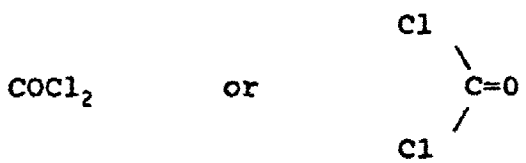


Figure 1

This is the chemical formula for phosgene, whose chemically active group is most likely the carbonyl group.

Phosgene is a particularly important substance from the chemist's point of view because of the highly reactive chlorine radical. Industrially, this substance is commonly used when chlorination is important in a chemical process.

Several of the important chemical properties of phosgene include a relatively low boiling point, resulting in a gaseous form at room temperature. Since its specific gravity is greater than air, it tends to hug the ground, as was observed in WWI, when the trenches were easily infiltrated by the substance. As a liquid, its specific gravity is also greater than water. Consequently, a liquid spill would be effectively covered by overlaying the liquid with water. Doing this would not result in a very active hydrolysis of this substance, a fact that becomes important later on. The solubility of phosgene in water is relatively slight. The gas has an odor similar to newly mown hay. Consequently, during WWI, it reminded the newly conscripted farm boys of home.

Historically, phosgene was first developed in 1812 by John Davy. He created it by combining carbon monoxide, chlorine, and a energy source (originally sunlight), hence the word phosgene. The substance was first utilized as a toxic agent at Flanders,

where a dense cloud released by the Germans from a series of canisters resulted in an initial exposure of perhaps 5000 individuals. As the soldiers became able to recognize and protect themselves against gas attacks, such a high exposure rate was never again achieved. During WWI, phosgene was rarely used by itself as an offensive agent. It was commonly combined with chlorine. Consequently, it is difficult to define how many WWI casualties were attributable to phosgene. Perhaps only 400 deaths occurred as a direct result of phosgene. Fairly high concentrations of phosgene are required to cause rapid debility and death, if the exposure is short-term. Within several months after the initial use by the Germans, the Allied Expeditionary Forces (AEF) began to use phosgene as well. By the end of the WWI, three to four times as much phosgene had been used by the AEF as by the Germans.

Shortly after the initial offensive use of phosgene during WWI, a major allied effort was undertaken to better understand and possibly treat the effects of this gas on individuals. Much of the early research was done by the British and most of this research was on the pulmonary effects. An early set of articles, which appeared as a training manual, demonstrated the clinical appearance of a gassed individual. Initially, an individual might be cyanotic as a result of acute hypoxia. Shortly thereafter, an individual might appear pale and hypotensive. Such observations were originally useful for triage, insofar as medical facilities during WWI were seriously compromised in their ability to deal with acute gas exposure. Although the basic physiology of shock and hypotension in this setting was not understood during WWI, we now know that this hypotension results from a combination of hypoxic acidosis and substantial volume loss in intravascular fluid into the pulmonary tissues. For purposes of triage, an individual at this stage of disease is generally not recoverable without extraordinarily sophisticated ICU care. In attempting to further understand exactly what phosgene does and how it affects the lung, animal studies were performed. For example, in a dog lung, subsequent to substantial exposure, the trachea appears intact and normal. The peripheral parenchyma, however, is highly inflammed and edematous. The primary effect of this agent is at the parenchymal level and not in the central airways.

Electron microscopic views of individual tissue exposed to phosgene show a smaller airway which is structurally normal and another, even higher view, shows epithelial tissue with cilia that are intact after an exposure.

Since phosgene is not primarily toxic to the airways, more peripheral studies were undertaken to determine the site of effect. For comparison, a histologic slide of normal tissue with

peripheral airways would show normal alveolar sac budding. Subsequent to a phosgene exposure, one sees the terminal portions of the airway walls beginning to become thickened. An electron microscopic view provides some additional information about the location of this change. First, a normal electron micrograph demonstrates that the tissues between the alveolar space and the interior of a capillary consist of multiple thin membranes. Subsequent to phosgene exposure, there is clear separation of those membranes. This is the site of first fluid leakage, which originates from the capillary, initially, into the pericapillary tissues (presumably through the cellular "tight junctions"). As the degree/quantity of leak increases, the tissues surrounding the larger blood vessels become edematous. As the interstitial tissues acquire more fluid, the lung becomes increasingly stiff and the patient perceives this as shortness of breath. That is to say, the initial sensation of abnormality is dyspnea. At this level of disease, the individual's own sense of respiratory limitation is a much more sensitive measure of his physiologic abnormality than are the techniques that we ordinarily bring to bear on increased lung water (i.e., stethoscope, chest x-ray, arterial blood gas). Furthermore, as the vessels become larger, more edema collects around the damaged vessel and, with substantial exposure (using a drawing from the WWI booklet), fluid begins to leak into the alveolar spaces, presenting classic pulmonary edema. Hence, we have the picture of a progressive pulmonary edema, a "noncardia" edema, that mimics ARDS. This picture could certainly be caused by a variety of other inhalation exposure, as well as phosgene.

In attempting to understand exactly how phosgene does this, a variety of studies have been undertaken to determine the characteristics of exposure. Phosgene seems to create its effects as a result of topical exposure. If a portion of the exposed animal's airway is plugged, the portion of lung peripheral to the plug does not suffer damage. If, as a result of a cross circulation experiment, one animal's blood is circulated into the vessels of a second animal and the first animal is then exposed to phosgene by inhalation, the second animal does not suffer symptoms of exposure. That is to say, the effects of phosgene are not blood borne. If phosgene is introduced intraperitoneally, it is not carried through the blood stream in substantial enough amounts to affect the lung.

Originally, it was felt that phosgene created its toxic effect as a result of hydrolysis. Therefore, phosgene would have been thought to produce substantial quantities of hydrochloric acid, creating its damage by direct acid toxicity. Thus, one would propose a similarity to the toxic pulmonary effects of gastric reflux. However, phosgene does not seem to have a substantial effect as a result of hydrolysis. First, the rate of

hydrolysis is actually quite low. Second, the buffering capacity of the lung is easily sufficient to deal with the small amount of hydrochloric acid that might be generated. Third, another substance which has an equivalent free carbonyl group (Ketene) produces effects nearly identical to phosgene without producing a hydrolytic acid byproduct. The carbonyl group ultimately damages proteins that maintain cell wall integrity, primarily those of the capillary tissues. Consequently, intravascular fluids leak through wall defects (or cell junction defects), the size of which depend largely on the degree of exposure.

Phosgene is a commonly available substance in this country. It can be found in many industrial settings other than military depots. U.S. industry annually uses six to eight times the amount of phosgene used during the entirety of WWI. Phosgene is a primary substance in the production of isocyanates; these substances being important precursors to a variety of foamed plastics. Phosgene is important in a variety of other chemical production schemes, largely because of the freely available chlorine radical. Destruction of foamed plastics readily produces phosgene (e.g., aircraft cabin fires). Firemen are at a particular risk of encountering this substance, as a result of the increased use of plastics that are susceptible to fires in home and industrial settings. Furthermore, phosgene is a primary precursor for a variety of insecticides, resins, and aniline dyes. All of these substances characterized by chlorinated hydrocarbon radicals may produce phosgene, if enough destructive energy is introduced (e.g., welding in an area of chlorinated hydrocarbon).

Pulmonary toxicity of phosgene depends on the ambient concentration of the agent, as well as the alveolar minute volume of the individual exposed. Levels above 80 to 90 parts per million (ppm) are rapidly fatal. Levels below 1 ppm produced minimal effects and those effects are produced only under conditions of prolonged exposure. There is probably no "safe" level where effects are possible, depending (to some extent) on pulmonary abnormalties that may already be present. It is important to understand that substantial toxicity can result from levels of 1.5 to 2 ppm. An individual without prior experience may not notice phosgene's aroma (newly mown hay) until levels of 2-3 ppm--hence, toxicity may occur without subject awareness. Carbon tetrachloride is an excellent example of a chlorinated hydrocarbon which, when heated, produces phosgene. As a result, its former popularity as a fire extinguisher has waned substantially. Methylene chloride (currently used as a paint stripping agent and available in local hardware stores) is also a problem from the perspective of chlorinated hydrocarbons which, when heated, produce phosgene. Companies manufacturing small machined metal parts often remove the grease from these parts by dipping

them in large tanks containing trichlorethylene and other halogenated hydrocarbons. The metal baskets used to dip these parts often require repair. Typically, welding of these baskets will be undertaken within the dipping tanks themselves (hopefully, after they have been thoroughly purged of residual vapors of the chlorinated hydrocarbons). Typically, the breakdown of chlorinated hydrocarbons by welding may produce a variety of degradation products. However, these are not sufficiently intense to act as sensory warnings to the worker.

EXAMPLE NO. 1

A 28-year-old individual, performing a welding repair of a dipping basket, had been welding in a particular location for approximately one half hour. At the end of the half hour, the individual felt somewhat dizzy, left his site of work, went to his supervisor, and was directed to the local hospital for evaluation of his dizziness. Patient was seen in the local hospital with an hour of onset of dizziness. A chest x-ray was taken and, having been interpreted as normal with a normal physical examination (particularly a normal chest exam), the individual was directed to return home. About a half hour after this set of studies, a thin watery sputum began to appear. Dyspnea progressed in severity and a repeat chest x-ray, taken three hours after the initial chest x-ray, showed substantial pulmonary edema. Swan-ganz catheter placement demonstrated that this individual's intracardiac pressures were within normal limits. Blood gases (room air) concurrent with the chest x-ray showed PO2 of 30, PC02 of 55, and intubation was undertaken. Three days after hospitalization, the sputum changed color and character. A chest x-ray showed a segmental pneumonitis and progressive respiratory failure ensured. A transbronchial biopsy was performed showing pneumonia - typical at this time, subsequent to exposure. Death finally ensued and the histologic section postmortem showed substantial interstitial fibrosis. It is suspected that such fibrosis is more likely due to chronic inflammation then to effects of phosgene.

EXAMPLE NO. 2

This 40-year-old furniture finisher had been working in a basement stripping furniture ("Zip Strip") on a cold, blustery, wintery day. As a result of this, all the basement windows had been closed and a small 1500 watt space heater had been used to provide heat. The patient felt mildly dizzy within an hour of commencing work, but she continued for an additional hour. Because of persistent dizziness and a strong sense of dyspnea, the patient presented herself to the local hospital approximately one half hour later. There a physical exam was within normal limits and a chest x-ray was performed and found to be normal.

Blood gases were obtained and found to show mild hypoxia (PO2 78). Because of a history of relatively sudden onset of dyspnea in a female also on birth control pills, a perfusion lung scan was performed. Abnormalities were identified, which were patchy and segmental in character, and an interpretation of pulmonary embolus was proposed. Immediate pulmonary angiography, however, showed normal pulmonary vasculature. Four hours after the first x-ray, substantial diffused bilateral infiltrates were observed and a better understanding of the individual's toxic inhalational exposure was obtained. Again, intracardiac catherterization was found to show normal pressures. Once again, the initial clinical investigation was remarkably normal with just as remarkable a rapid onset of abnormal physiology.

The therapy of toxic inhalations is generally categorized as Prevention, Prophylaxis, Postexposure chemical interference with the exposing agent, and Postexposure clinical treatment of the physiologic effects of the agents.

Prevention of toxic inhalations can be undertaken via education, provision of a protective environment, and substitution of "nontoxic" substance.

Prophylaxis of toxic inhalation can occur as a result of chemical binding of the agent, induction of degrading enzymes for the agent, or chemical blocks of active receptor sites for the agents. A variety of substances have been studied with respect to the carbonyl group of phosgene. Of these, mandelamine (HMT) is the most active. In animal studies, this substance is a relatively effective prophylactic at high doses. However, this substance must be introduced prior to exposure and at dose levels that would not be practical for chronic use. Some clinical experience using this substance in patients who have been exposed to phosgene has not been convincingly successful.

Postexposure chemical treatment has not been thoroughly studied. HMT does not seem to be a useful agent here. The use of steroids (even in "industrial" doses) seems useful only if begun some four to six hours prior to exposure.

Much of the effort directed toward exposures today is directed toward postexposure clinical treatment. The areas of concern here include: pulmonary edema, hypoxia, hypotension, secretions, bronchospasm, right heart failure, and infection.

The pulmonary edema, observed subsequent to toxic inhalation exposure, has been treated by a number of techniques. Reduction of pressure in the pulmonary artery appears to be of some value. Consequently, patients are maintained mildly dehydrated (with attention to the effects of minimized intravascular volume, if

positive pressure ventilation is necessary). Some attempts to replace surfactant have generally met with failure. Control of the capillary leak in animal studies has been possible through the use of long-chain molecular plastics (poly vinyl pyrrolidines). This approach is far from ready for human clinical trial. Positive pressure ventilation has a measurable affect on the rapidity and severity of capillary leak and should probably be instituted early.

The hypoxia seen in this setting is generally thought to be the result of severe combined V.O. and diffusion defects. Oxygen supplementation generally suffices with positive pressure ventilation occasionally necessary.

Secretions are typically thin watery initially and the use of atropine-like substances is generally of minimal benefit.

Hypotension is commonly seen as a result of a combination of hypoxia related acidosis and depleted intravascular volume (leak into pulmonary interstitium). There is little evidence to suggest that colloid therapy is preferable to crystalloid therapy.

Bronchospasm is occasionally seen, but usually as a late presentation. Often, individuals presented with bronchospasm fall into the catagory of "closet" asthmatics: individuals with irritating airways diseases whose peribronchial smooth muscle shows increased tone because of increased interstitial fluids (similar to cardiac asthma).

Right heart failure is typically seen and may be the result of a combination of hypoxia, acidosis, and positive pressure necessary to ventilate.

Infection will often supervene, with first signs occurring at day three to day four, subsequent to exposure. Routine antibiotic prophylaxis is clearly not effective in preventing superinfection.

EXAMPLE NO. 3

This 46-year-old individual had suffered a house fire exposure with previously normal pulmonary function. Fire, particularly fires in closed spaces and in settings where substantial plastics are burned, often produce combination inhalation exposures with phosgene a major contributing factor. Subsequent to a fire exposure, a bronchoscopy is performed to assess the degree of edema of the upper airway and trachea. This assessment allows early anticipation of postexposure pharyngeal edema. It is important to understand that toxic exposure is the

result of the gases and fumes created by the fire, rather than the temperature of the air itself. A chest x-ray on the first day, in this individual, showed only faint infiltrates in the peripherery. The second day there were substantial peripheral infiltrates which, by the third day went on to become increasingly prominent, as a result of superinfection. This, again, is a typical clinical presentation and time course of toxic inhalation.

EXAMPLE NO. 4

This 18-year-old individual has been part of a group of soldiers exposed to a confined space release of HC (military smoke). He became short of breath and was evaluated in a local emergency room. There he was found to have a normal physical examination and subsequently discharged. Upon return several hours later, he was moderately dyspneic, with a chest x-ray which showed diffuse infiltrates, typical of the delayed effects of phosgene exposure.

In summary, phosgene exposure, taken by itself and taken as an example of a wide variety of toxic inhalation exposures, requires careful, thoughtful consideration of the fact that the patient is often clinically aware of his limited pulmonary function and impeding respiratory disaster long before medical personnel are able to corroborate his complaint by physical exam, x-ray, or arterial blood gas (ABG) measurement. Typically, after exposure, there may be a "lag period" of four to six hours before substantial changes will be evident by physical examination, ABG, or chest x-ray. Certainly, substantial changes may occur even later than that, but they are increasingly less likely to be life-threatening if their onset is further delayed from the acute exposure.

When faced with an acute exposure, the following is strongly recommended:

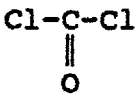
1. Isolation from the acute exposure.

2. Immediate response to an individual's sense of dyspnea by physical examination. If this is normal (that is no wheezes on forced expiratory maneuver) and if the clinical situation permits, this individual must be observed in a controlled setting for an additional four to six hours postexposure. At that time a repeat clinical examination, arterial blood gas, and chest x-ray would be appropriate. If all three measurements are within normal limits, the individual may be safely discharged with the knowledge that he is unlikely to develop a disease severe enough to lead to death. If any one of these parameters is abnormal,

either initially or at the four to six hour review, the individual should be closely monitored for the ensuing 24 to 48 hours, preferably in an intensive care setting. Pulmonary dysfunction can progress exceptionally rapidly once early signs of pulmonary edema are present.

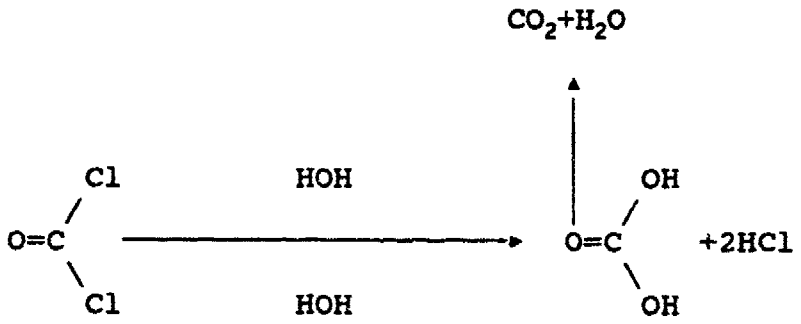
Again, an individual's sense of dyspnea usually appears long before our clinical ability to confirm abnormal physiology. At this point, longitudinal observation is necessary.

PHOSGENE CHEMISTRY



Also known as: Carbonyl Chloride
Carbon Oxchloride
Chloroformyl Chloride

PHOSGENE HYDROLYSIS



This is a low rate reaction and <u>not</u> a major factor in toxicity of phosgene.

PHOSGENE ACYLATION

Where X = NH-, NHNH-, -NR-, -O-, -S-
R = N, aryl, aklyl

This reaction seems to account for the major toxic effects of phosgene. The acylation of various proteins destabilized cell functions with loss of cellular wall integrity and consequent (in lung) plasma leakage (from damaged capillaries) into the pulmonary interstitium - producing pulmonary edema pattern.

PHOSGENE TOXICITY

1. Effects of phosgene are due to topical exposures

   a. bronchial plugging protects free local toxicity
   b. cross circulation does not transmit disease
   c. interperitoneal injection does not injure lung

2. HCl is not the specific agent

   a. calculated buffering capacity of the lung should protect against HCl released
   b. Ketene (has C=O group but does not produce HCl) appears as toxic as phosgene

3. C=O group functions as an acylating agent

SUBSTANCES BINDING -CO GROUP OF PHOSGENE

Name	% bound	Yeast protection
HMT	80+	Complete
PABA	70	Complete
Taurine	60	Fair
Sulfanilic acid	45	Fair
Urea	0	None
Na Thiosulfate	0	None
Coagulin	20	Complete

PHOSGENE EXPOSURE
ACUTE TREATMENT

1. Remove from contamination
2. Administer artificial respiration
3. Evaluate by CXR, ABC, auscultation
4. Monitor closely for 24-48 hours

Note that CXR, ABC, auscultation may be normal up to 4 hours after exposure with individual still succumbing to the exposure within 48 hours.

Therefore, perform <u>each</u> of CXR, ABC, auscultation initially <u>and/or at least</u> at 4-6 hours before certifying patient unlikely to die of exposure. If an abnormality is identified the individual must be monitored for 24-48 hours. Even after 4-6 hours of "normal" CXR, ABC, auscultation, disease may appear ("delayed" effect) but is unlikely to be lethal.

THERAPY OF TOXIC INHALATIONS

1. Prevention
2. Prophylaxis
3. Post-exposure chemical interference with agent (presumes chemical latent period)
4. Post-exposure clinical treatment (presumes clinical changes are "bad")

PREVENTION OF TOXIC INHALATION

Education
Protective environment
Substitute "non-toxic" substances

PROPHYLAXIS OF TOXIC INHALATION (Phosgene)

Carrots (Retinoids)	Vitamin K
Steroids	Thymoxyethyldimethylamine
Magnesium carbonate	Dehydration
Thromboplastin	Phosgene pretreatment
HMT	

All of the above approaches have some measurable prophylactic effect on phosgene-exposed animals.

POST-EXPOSURE CHEMICAL TREATMENT

HMT - Poor data @ 4 gm IV
STEROIDS - may decrease mortality if used in pretreatment as well

POST-EXPOSURE
CLINICAL TREATMENT

1. Pulmonary edema
2. Hypoxia
3. Hypotension
4. Secretions
5. Bronchospasm
6. Right heart failure
7. Infection

POST-EXPOSURE
CLINICAL TREATMENT I

1. Pulmonary edema: pressure reduction (e.g., diuretic)
   surfactant replacement
   capillary leak
   ventilator pressure (positive pressure/PEEP)

2. Hypoxia - combined V/Q and diffusion

3. Secretions: reduction
   thinning
   clearance

4. Hypotension - plasma replacement not effective

POST-EXPOSURE
CLINICAL TREATMENT II

5. Bronchospasm inflammatory if high dose phosgene (steroids may help)

6. Right heart failure: dilatation of right heart
   venous engorgement
   nl wedge pressure

7. Infection - prophylaxis not effective

DISCUSSION AFTER DR. URBANETTI'S PRESENTATION

Dr. Fowler brought up the subject of bronchiolitis obliterans as a long-term complication of this type of gas exposure, noting that if it occurred, one would not expect the frequency-dependent compliance changes to normalize, nor the DLCO changes to normalize. Dr. Urbanetti responded that the literature on bronchiolitis obliterans was outdated, and that this condition was not a typical consequence of phosgene exposure. He added that it was difficult to differentiate the effects of superinfection from the "pure disease effects," and that he did not have any biopsies from survivors of phosgene exposure.

Dr. Assaad brought up the subject of surfactant and positive airway pressure, noting that surfactant levels may be affected as the edema progresses. In regard to the use of positive airway pressure, he inquired whether the beneficial effect involved a decrease in the rate of alveolar secretions or a decrease in inflammatory secretions. In response, Dr. Urbanetti said that in his experience, the application of positive airway pressure was useful therapy, although he could not comment on what mechanisms may have been involved.

Further discussion centered on special cases and types of therapy. For example, Dr. Urbanetti commented that he did not think steroid treatment was particularly useful, noting that, because very little data was available regarding various types of treatment modalities, a well-founded assessment could not be made. He said that individuals who had preexisting pulmonary dysfunctions such as asthma might be more prone to complications such as right heart failure. Another point discussed by Dr. Urbanetti and MAJ Ripple was the importance of rest in the prevention of infection. Resting the patient was known to be quite beneficial since exercise and vigorous body movements--by virtue of the increased cardiac output and related effects on capillary pressure and systemic hypoxia--worsens the pulmonary condition after toxic gas exposure.

INVESTIGATION OF ACUTE LUNG INJURY

Dr. Sami Said

This presentation consisted of an overview of the factors involved in the onset, development, and pathophysiology of acute lung injury. Dr. Said indicated that the investigation of acute lung injury can be divided into a number of components, which collectively summarize the progression of injury. Each component requires a response on the part of the clinician or can be addressed by a researcher: (1) it is important to recognize the potential for acute lung injury after a respiratory insult; (2) the severity of the injury should be graded; (3) the onset of injury should be anticipated and detected; (4) triggering events must be identified; (5) the sequence of events leading to injury and the progression of injury must be elucidated; and (6) morbidity and mortality must be reduced. Future research should concentrate on the major knowledge gaps, which include the current inability to quickly detect the onset of injury or to reverse injury after it has occurred. Also, much remains to be determined regarding the cellular mechanisms of action leading to injury.

Each of the topics listed above was discussed, and the current state of knowledge in each area was outlined.

INVESTIGATION OF ACUTE LUNG INJURY

Dr. Sami Said

A WISH LIST

1. Recognize its presence.
2. Grade its severity.
3. Detect its onset.
4. Identify the triggering event.
5. Unravel the sequence of events.
6. Reduce its morbidity and mortality.

WHAT CAN BE ACCOMPLISHED NOW

1. Recognize its presence.
2. Grade its severity.
3. Some understanding of mechanisms.
4. Attenuate injury before it occurs.

STILL TO BE ACHIEVED

1. Detect or anticipate onset of injury.
2. Identify its triggering event.
3. Reverse injury after it sets in.

EVALUATION OF ACUTE LUNG INJURY

A. PULMONARY EDEMA (LUNG WATER)

1. Wet lung weight: related to body weight (corrected for blood), or to dry weight.

2. Macroscopic appearance: lungs enlarged, liver-like, hemorrhagic or consolidated; frothy liquid and foam in airways.

3. Pulmonary lymph flow rate: (sheep or dogs).

B. PERMEABILITY

1. MICROVASCULAR

   a. May be abnormal before lung water content increases.

   b. Separates high-permeability from high-pressure edema.

2. VASCULAR (& EPITHELIAL)

   a. Bronchoalveolar lavage: usually as a terminal procedure. Examined for:

      (1) Cells: total count, shifts in proportions of macrophages, granulocytes, lymphocytes and others

      (2) Albumin, globulins, and other serum proteins

      (3) Enzymes: LDH, alk. phosphatase, proteases, could help identify cellular source of injury.

   b. Clearance of labeled solutes (e.g., DTPA, albumin) from blood into bronchoalveolar secretions; relative clearance of small and large molecules is a sensitive indicator of A-C barrier function.

   c. Protein flux in pulmonary lymph: lymph/plasma albumin and globulin ratios (sheep and dogs).

C. EVIDENCE OF INJURY

1. MORPHOLOGIC EXAMINATION:

   a. Light microscopy: Can confirm airway damage and diffuse parenchymal injury (peribronchial and perivascular cuffs, intra-alveolar edema, inflammatory cell infiltration, RBC extravasation).

   b. EM: Important for defining cellular site of injury, but focal nature of lesions poses sampling problem.

   c. Other: Morphometry, autoradiography, histochemistry.

2. FUNCTIONAL EVIDENCE

   a. Impaired endothelial cell function

      (1) Decreased uptake of 5-HT or PGE.

      (2) Release of ACE.

      (3) Loss of (endothelium-dependent) relaxation by acetylcholine.

   b. Alveolar epithelial cell function: decreased uptake of polyamines (especially putrescine), after injury with chemicals such as paraquat, trialkyl phosphorothioates, and O_2.

D. EARLY DETECTION/MARKERS OF EARLY INJURY

1. Should permit prompt and more effective treatment.

2. Markers must be sensitive, specific, and relatively easy to measure.

3. Detection of "at risk" state?

4. Proposed biochemical markers of acute lung injury.

   a. Products of complement activation
   b. Factor VIII-related antigen
   c. Fibronectin
   d. Lipid peroxide
   e. Pentose pathways enzymes
   f. Surfactant impairment

PATHOGENESIS OF ARDS

MECHANISMS OF LUNG INJURY

1. Direct: as by smoke, toxic gases, aspiration of HCL.
2. Indirect, i.e., mediated by endogenous toxic agents: important in most other conditions.

ARDS: ALTERED PHYSIOLOGY

1. Progressive, refractory hypoxemia.
2. Lungs progressively less compliant, i.e., increasingly difficult to inflate, except at very high pressures.
3. Grossly increased venous admixture (shunt), dead space and work of breathing.
4. Alveolar ventilation (Pa_{CO2}) remains normal, though at considerable cost to patient.

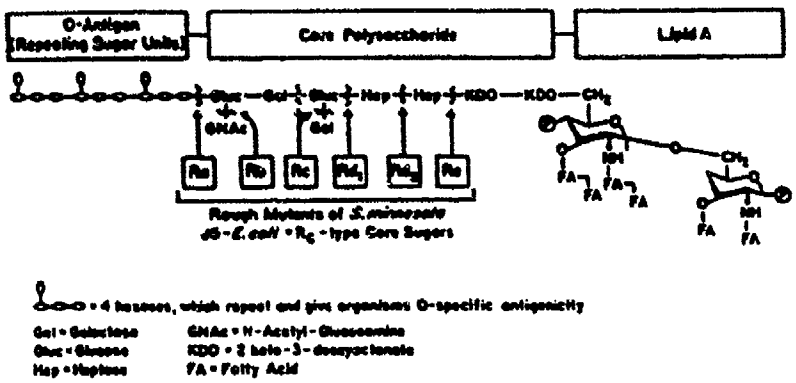
SEPTIC SHOCK

PATHOGENESIS

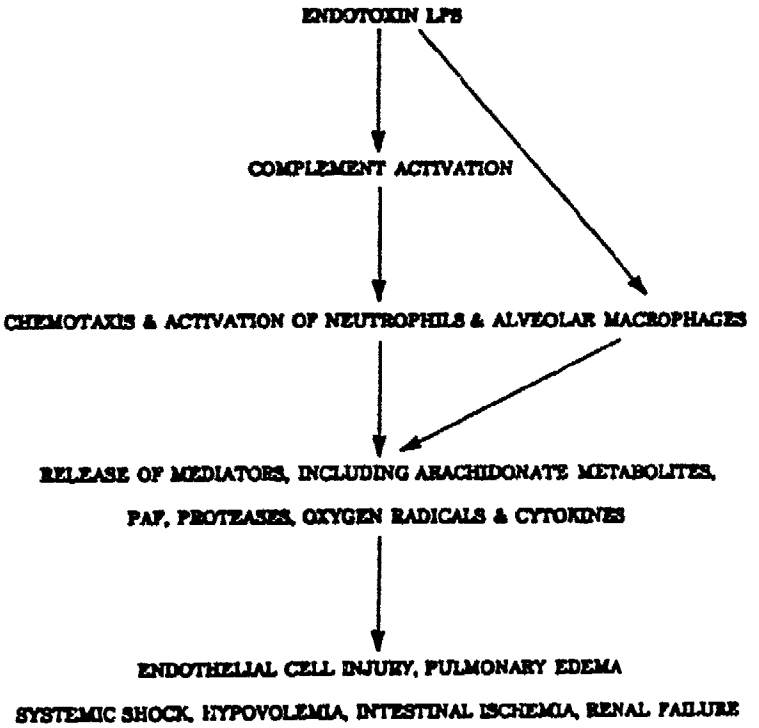
1. The lipopolysaccharide (LPS) component of gram-negative endotoxin is the main determinant of pathology in gram-negative bacteremia.

2. LPS elicits the host responses through a variety of mechanisms and mediators.

THE STRUCTURE OF LIPOPOLYSACCHARIDE



POSTULATED STEPS IN THE PATHOGENESIS OF ACUTE LUNG INJURY DUE TO GRAM-NEGATIVE SEPSIS



ALTERNATE PATHWAYS FOR LUNG INJURY IN ENDOTOXIN SHOCK

1. LPS stimulates neutrophils directly,
2. LPS injures endothelium directly,
3. LPS induces alveolar macrophages to generate TNF/cachectin.

MEDIATORS OF ACUTE LUNG INJURY

1. Arachidonic acid metabolites, especially thromboxane A_2, leukotrienes and lipoxins
2. Platelets and platelet-activating factor (PAF)
3. Complement
4. Tumor necrosis factor (cachectin)
5. Oxygen-free radicals
6. Granulocytes and macrophages

THROMBOXANE A_2

1. Pulmonary TX synthesis is stimulated during acute lung injury.
2. TX contributes to pulmonary hypertension and decreased pulmonary compliance due to *E. coli* endotoxin in sheep.
3. Inhibition of Tx synthesis protects against many features of acute lung injury, though usually not against high-permeability pulmonary edema.

LEUKOTRIENES

1. Cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) induce airway and pulmonary vasoconstriction, and increase microvascular permeability.
2. These LT's also cause an initial rise in B.P. (due to vasoconstriction), followed by hypotension, due to generalized leakage of plasma from microvessels and decreased cardiac contractility.
3. LTB_4 stimulates leukocyte migration, aggregation and adherence to endothelium.

LIPOXINS

1. Lipoxins A and B (LXA, and LXB) are produced by human leukocytes through the action of 5- and 15-lipoxygenases on arachidonic acid (lipoxygenase interaction products).

2. LXA stimulates neutrophil chemotaxis and superoxide anion generation; contracts guinea pig lung strip; and dilates systemic microvessels, but does not increase their permeability.

PLATELETS AND PLATELET-ACTIVATING FACTOR (PAF)

1. Platelets have been thought to contribute to lung injury, because platelet aggregation is often associated with the injury.

2. But recent evidence suggests that normal platelets may help maintain pulmonary vascular integrity.

3. PAF

   a. Induces platelet & granulocyte aggregation.

   b. Also causes pulmonary vasoconstriction, airway constriction, systemic hypotension and pulmonary edema in several animal species.

   c. Specific assay for PAF not yet available, so it's possible release in ARDS hasn't been measured.

   d. But specific antagonists are now available and are being used to evaluate its role in lung injury.

COMPLEMENT

1. $C5_a$ generated from endotoxin- or zymosan-activated plasma causes neutrophil aggregation *in vitro*.

2. $C5_a$-activated neutrophils are more adherent and are cytopathic to endothelial cells in culture.

3. Mice genetically deficient in $C5_a$ do not develop pulmonary edema in response to pneumococcal sepsis, burns, or hyperoxia.

4. Anti-C5a antibodies prevent pulmonary edema and hypoxemia, and improve survival in primates with severe *E. coli* sepsis.

TUMOR NECROSIS FACTOR (CACHECTIN)

1. A polypeptide produced in large quantities by endotoxin-activated macrophages.

2. Injection of TNF in rats reproduces features of severe endotoxin shock: hypotension, metabolic acidosis, hemoconcentration, pulmonary edema, and hemorrhage, bowel ischemia, acute renal tubular necrosis and death.

OXYGEN-FREE RADICALS

1. Active species of oxygen are among the principal mediators of acute lung injury. Cytotoxic O_2 species include:

$O_2\cdot$	Superoxide radical
H_2O2	Hydrogen peroxide
$OH\cdot$	Hydroxyl radical
$^1O_2$	Singlet oxygen
$ROO\cdot$	(R = Lipid)

2. The O_2 metabolites are produced by activated granulocytes and alveolar macrophages.

3. Experimental generation of free radicals leads to acute pulmonary edema *in vivo* and *in vitro*.

4. Increased oxidant activity is present in bronchoalveolar lavage fluid from ARDS patients.

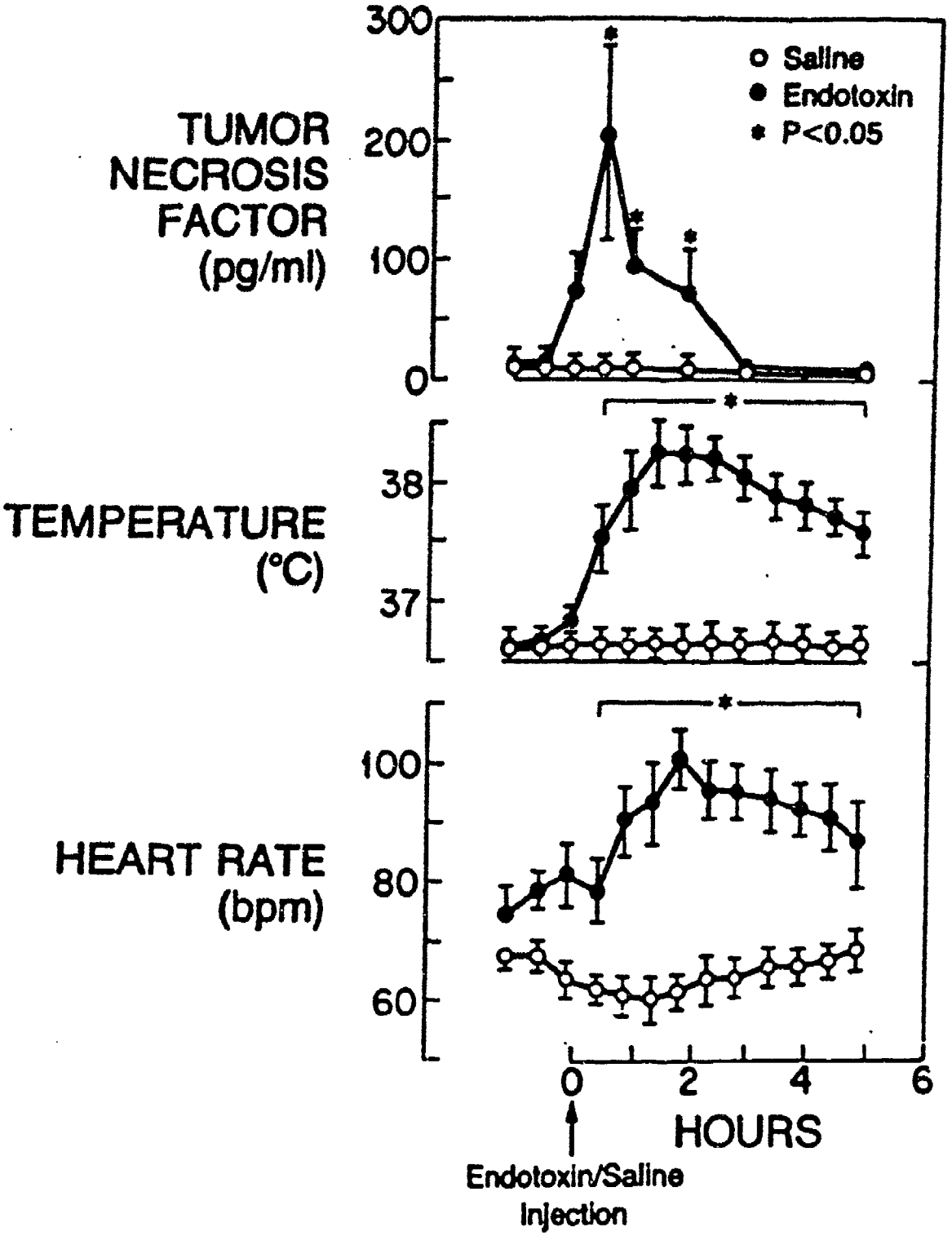
5. Pretreatment with free-radical scavengers protects against pulmonary edema in several experimental preparations.

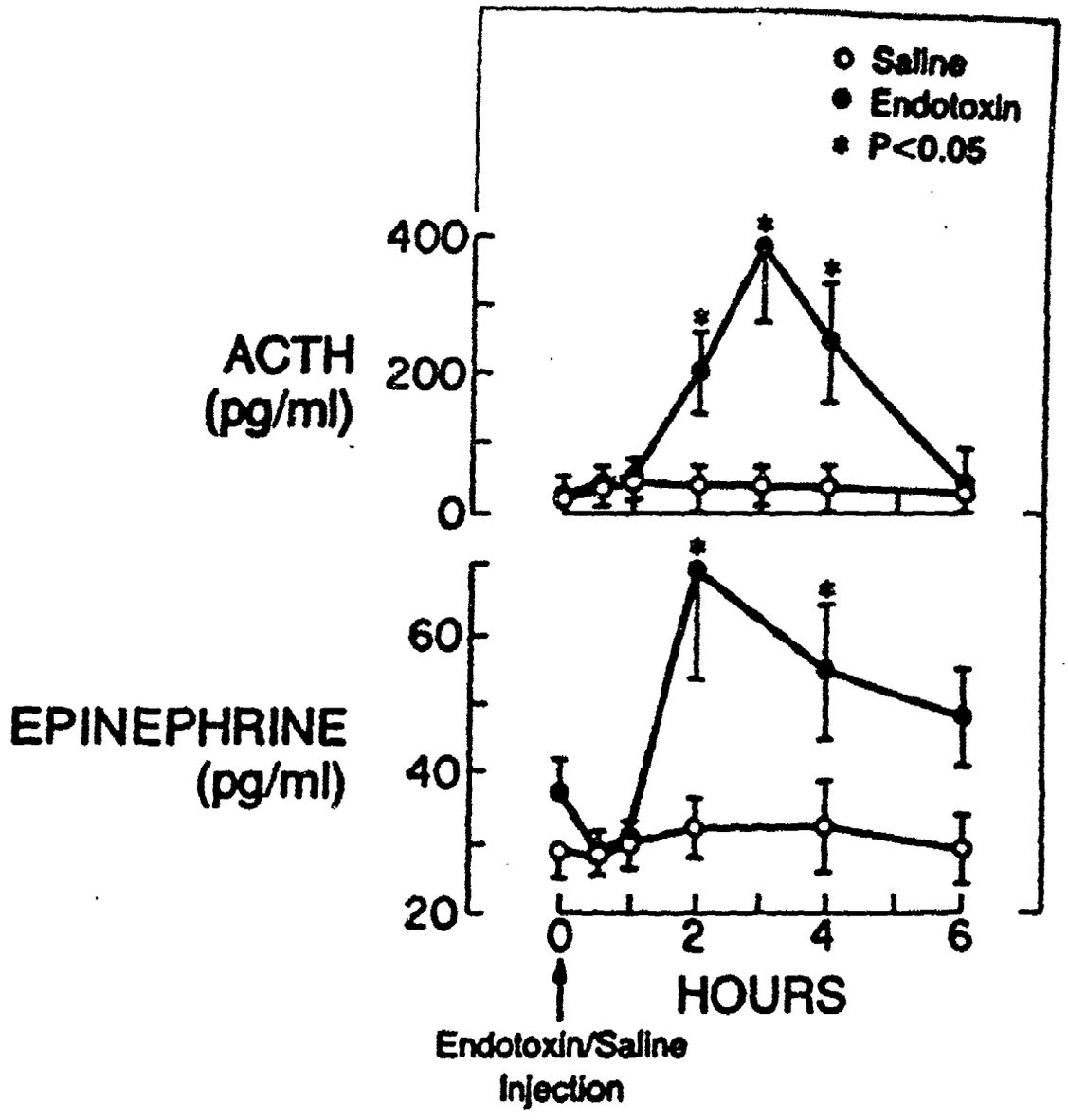
6. Anti-oxidant defenses

   a. Multiple oxidants, including free oxygen radicals, are normally generated in the lung and are present in the environment.

   b. Diverse mechanisms are available for their detoxification.

   c. Cell and tissue injury results when these defense mechanisms are overwhelmed.


TUMOR NECROSIS FACTOR (pg/ml)
300
200
100
0
Saline
Endotoxin
* P<0.05
TEMPERATURE (°C)
38
37
HEART RATE (bpm)
100
80
60
0
2
4
6
HOURS
Endotoxin/Saline Injection


Saline
Endotoxin
* P<0.05
ACTH
(pg/ml)
400
200
0
EPINEPHRINE
(pg/ml)
60
40
20
0
2
4
6
HOURS
Endotoxin/Saline
Injection

GRANULOCYTES

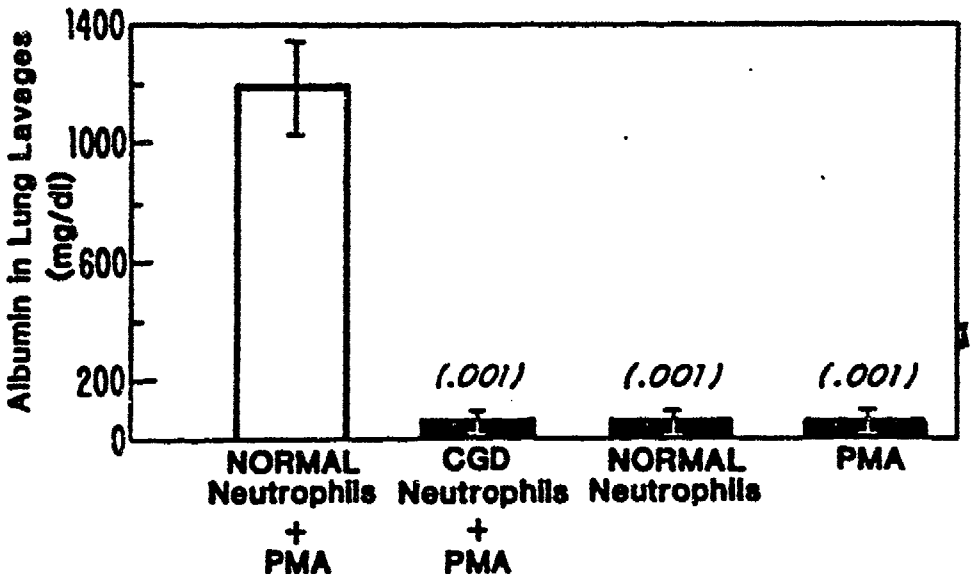
1. In ARDS, granulocytes are sequestered in pulmonary microvessels and migrate into interstitial and alveolar spaces.

2. Upon stimulation, normal granulocytes release free radicals, proteolytic enzymes, arachidonic acid metabolites and platelet-activating factor.

3. Granulocytes with impaired function (from CGD patients) cannot induce pulmonary edema in isolated lungs.

4. Depletion of granulocytes protects against lung injury in animal models.

5. ARDS can occur in patients with severe neutropenia.



ALVEOLAR MACROPHAGES

1. Secrete a variety of factors (lymphokines or monokines) that attract and activate granulocytes.

2. Like granulocytes, macrophages may also secrete mediators such as PAF, arachidonate metabolites and free radicals and proteases.

3. Stimulated by endotoxin, macrophages produce the highly toxic polypeptide cachectin (tumor necrosis factor).

INTERACTIONS AMONG MEDIATORS

1. Activation of cells is essential for release of soluble mediators and oxygen radicals.

2. Reactive O_2 species trigger arachidonic acid release, and metabolism of AA produces oxygen radicals.

3. PAF and AA products have synergistic effects on platelet aggregation and neutrophil activation.

4. PAF promotes release of cytokines and stimulates acetyl transferase, a key enzyme in PAF biosynthesis.

5. gamma-Interferon enhances TNF production and its biological response.

6. IL-2 induces TNF synthesis by human blood monocytes and alveolar macrophages.

FEATURES OF INFLAMMATION/INJURY PREVENTED OR REDUCED BY VIP

1. Airway constriction.
2. Pulmonary vasoconstriction.
3. Increased wet weight of lung.
4. Increased vascular permeability.

VIP AS AN ENDOGENOUS MODULATOR OF INFLAMMATION

CRITERIA TO BE FULFILLED:

1. Should be capable of preventing or reducing inflammation.
2. Should be released in response to inflammation.
3. Its inhibition should aggravate the inflammatory reaction.

RELEASE OF VIP IN RESPONSE TO ACUTE INJURY

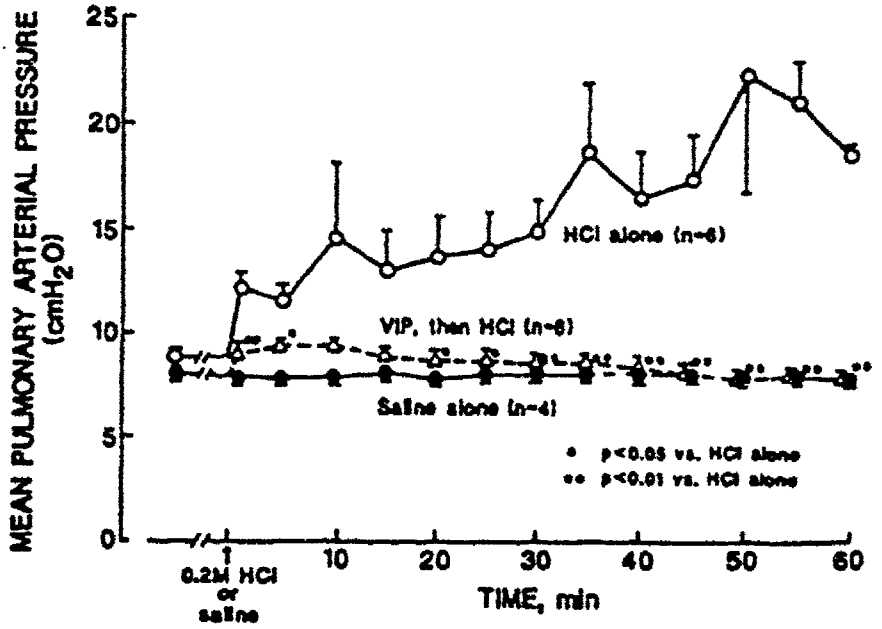
1. Plasma VIP levels increased 10-fold after i.v. infusion of PAF in dogs.
2. VIP concentration in pulmonary perfusate increased 22-fold following oxidant injury with xanthine-xanthine oxidase.

ANTI-INFLAMMATORY EFFECT OF VIP

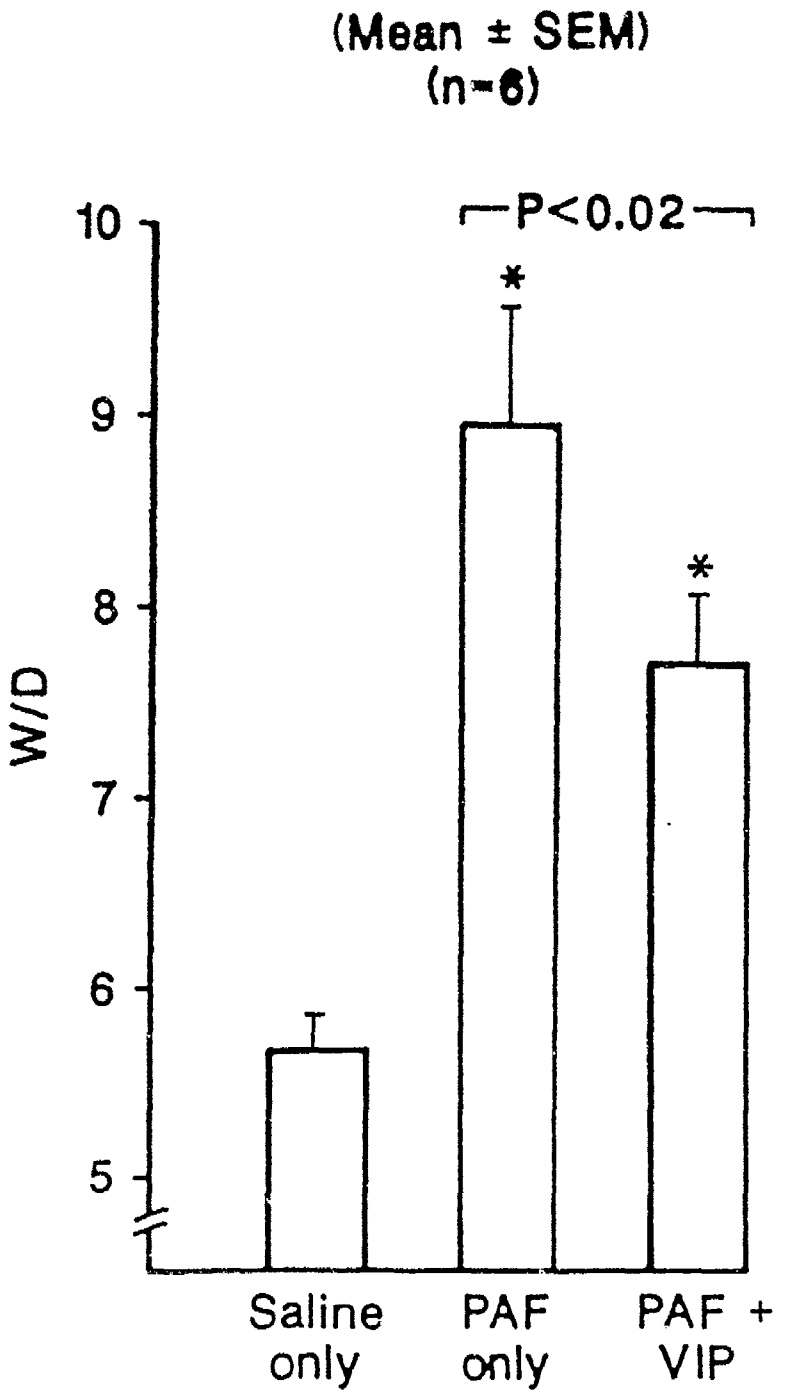
POSSIBLE MECHANISMS:

1. Stimulation of cyclic AMP production (?).
2. Vasodilation (?).
3. Inhibition of release of cyclooxygenase and lipoxygenase products.
4. Inhibition of inflammatory cells and their release of cytokines.
5. Antagonism of actions of proinflammatory mediators (histamine, $PGF_{2\alpha}$, PAF, neurokinins).
6. Anti-oxidant activity.

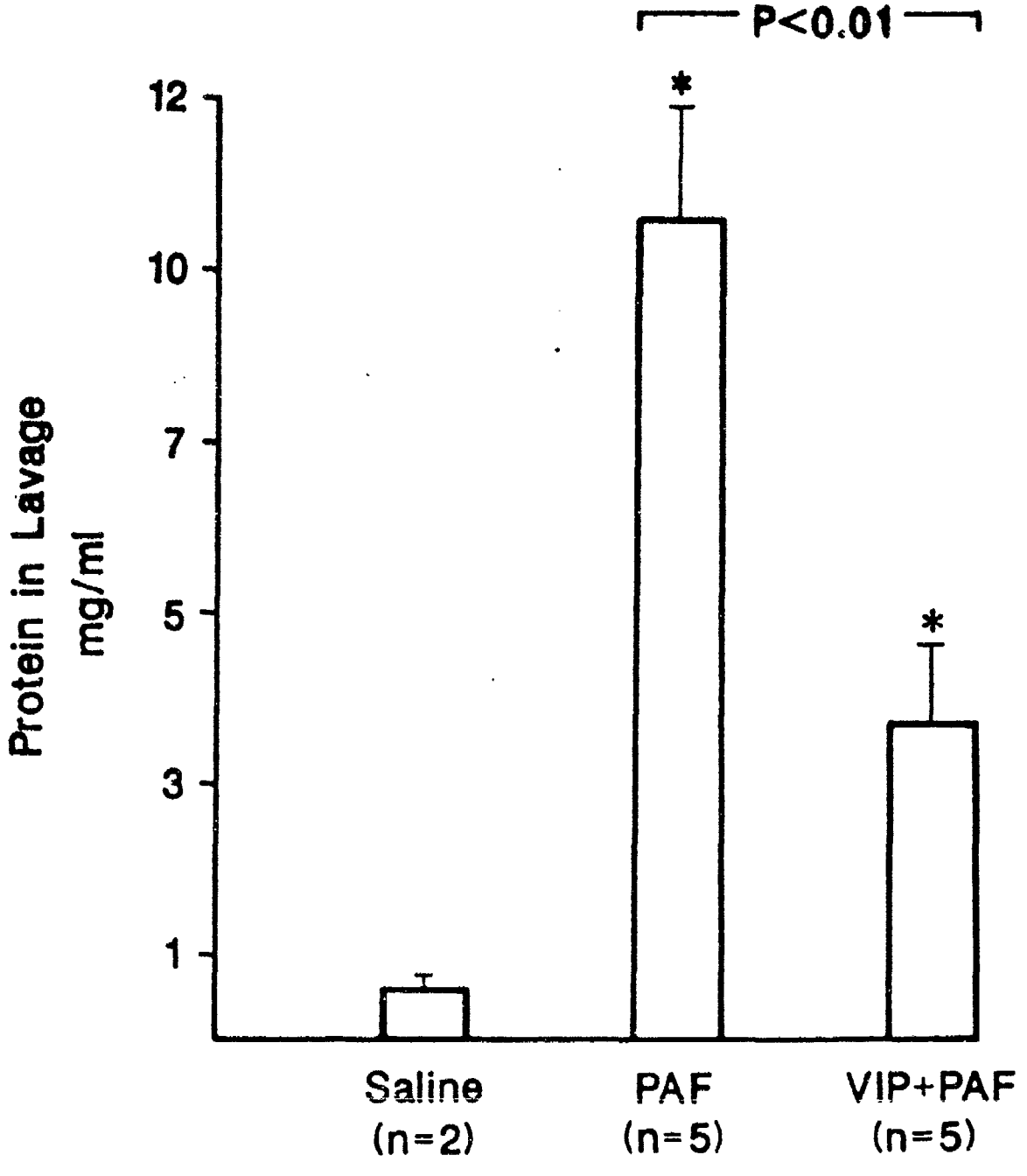
VIP PREVENTS THE RISE IN PULMONARY ARTERY PRESSURE AFTER HCL

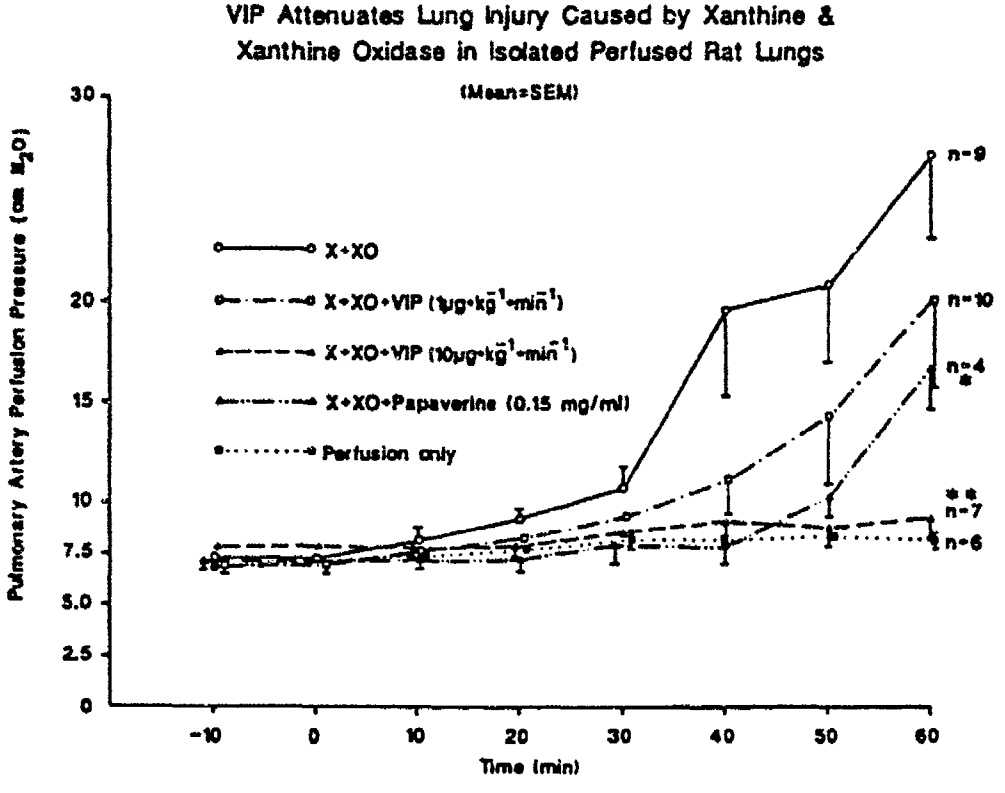


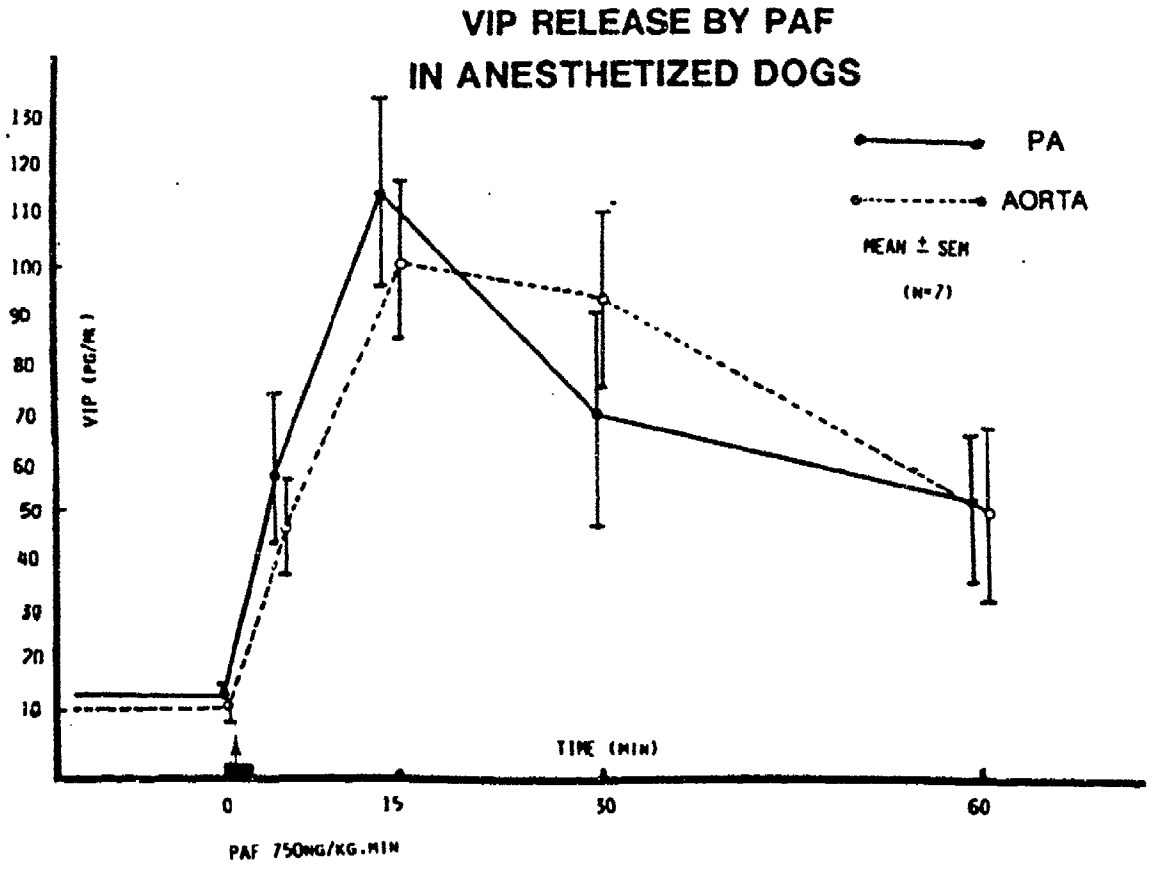
VIP PROTECTS AGAINST PULMONARY EDEMA INDUCED BY PAF IN PERFUSED RAT LUNG



VIP DECREASED PROTEIN CONTENT IN BRONCHOALVEOLAR LAVAGE FLUID FOLLOWING ADDITION OF PAF TO PERFUSATE OF RAT LUNGS




VIP Attenuates Lung Injury Caused by Xanthine & Xanthine Oxidase in Isolated Perfused Rat Lungs
(Mean±SEM)
Pulmonary Artery Perfusion Pressure (cm H_2O)
30
20
15
10
7.5
5.0
2.5
0
-10
0
10
20
30
40
50
60
Time (min)
X+XO
X+XO+VIP (1μg·kg⁻¹·min⁻¹)
X+XO+VIP (10μg·kg⁻¹·min⁻¹)
X+XO+Papaverine (0.15 mg/ml)
Perfusion only
n=9
n=10
n=4
n=7
n=6
*
* *


VIP RELEASE BY PAF
IN ANESTHETIZED DOGS
PA
AORTA
MEAN ± SEM
(N=7)
VIP (PG/ML)
150
120
110
100
90
80
70
60
50
40
30
20
10
TIME (MIN)
0
15
30
60
PAF 750NG/KG.MIN

DISCUSSION AFTER DR. SAID'S PRESENTATION

COL Dunn raised the issue of granulocyte dependence in the various models used in this area of research, and asked specifically about the xanthine-xanthine oxidase model. Dr. Said responded that he did not think that granulocytes were important in that particular system and noted that in his laboratory, they routinely washed the excised lungs very carefully and that they don't expect to find many granulocytes in that type of isolated lung preparation. He added that there are some tissue monocytes, lymphocytes, and macrophages present, however, noting that one of these three cell types may be more important than the granulocytes (probably the macrophages) in the injury mechanism.

COL Dunn stated that the current state of knowledge suggests that the organofluorine-induced lung lesion is not dependent upon granulocyte involvement. Thus, any mechanism involving granulocyte participation, or inhibition of granulocyte products, was probably not relevant. Dr. Said concurred, noting that it was recently reported that eosinophils contained high amounts of VIP and they may have a role in this type of injury.

BIOCHEMICAL MARKERS OF ORGANOHALIDE-INDUCED PULMONARY EDEMA AND ACUTE LUNG INJURY

Dr. Ayaad W. Assaad

The mechanisms of action of the organohalides and phosgene are not fully understood. Thus, this preliminary study was performed to test an hypothesis which proposed that inhalation of organohalides was followed by the generation of arachidonic acid metabolites via the lipoxygenase and cycloxygenase pathways and, that as a consequence, factors such as thromboxane, prostaglandins, and leukotrienes would lead to increased pulmonary vascular and bronchial constriction and increased pulmonary microvascular permeability.

Unanesthetized sheep were exposed for 10 minutes to toxic doses of PFIB or TFD; controls were exposed to room air. Blood samples were collected at various intervals and assayed by RIA for thromboxane and for 6-keto-PGF_{1a} (the most stable metabolite of prostacyclin).

Results indicated that PFIB and TFD act through different mechanisms to induce acute lung injury and pulmonary edema. TFD appeared to cause a primary lesion in the pulmonary endothelial cell membrane, and evidence suggested that arachidonic acid metabolites were involved: both thromboxane and 6-keto-PGF_{1a} were significantly elevated (approximately sevenfold) 10 minutes after exposure.

It was concluded that specific inhibitors to enzymes in the arachidonic acid cascade may be of therapeutic value in attenuating clinical symptoms of pulmonary intoxication associated with exposure to organohalides.

BIOCHEMICAL MARKERS OF ORGANOHALIDE-INDUCED PULMONARY EDEMA AND ACUTE LUNG INJURY

Dr. Ayaad W. Assaad, LTC Jill R. Keeler,
LTC James B. Nold, and MAJ David H. Moore

U.S. Army Medical Research Institute of Chemical Defense
Pathophysiology Division
Aberdeen Proving Ground, Maryland

ARACHIDONIC ACID METABOLITES AND THEIR POSSIBLE ROLE IN THE PATHOGENESIS OF ORGANOHALIDE-INDUCED ACUTE LUNG INJURY AND PULMONARY EDEMA: A PRELIMINARY STUDY

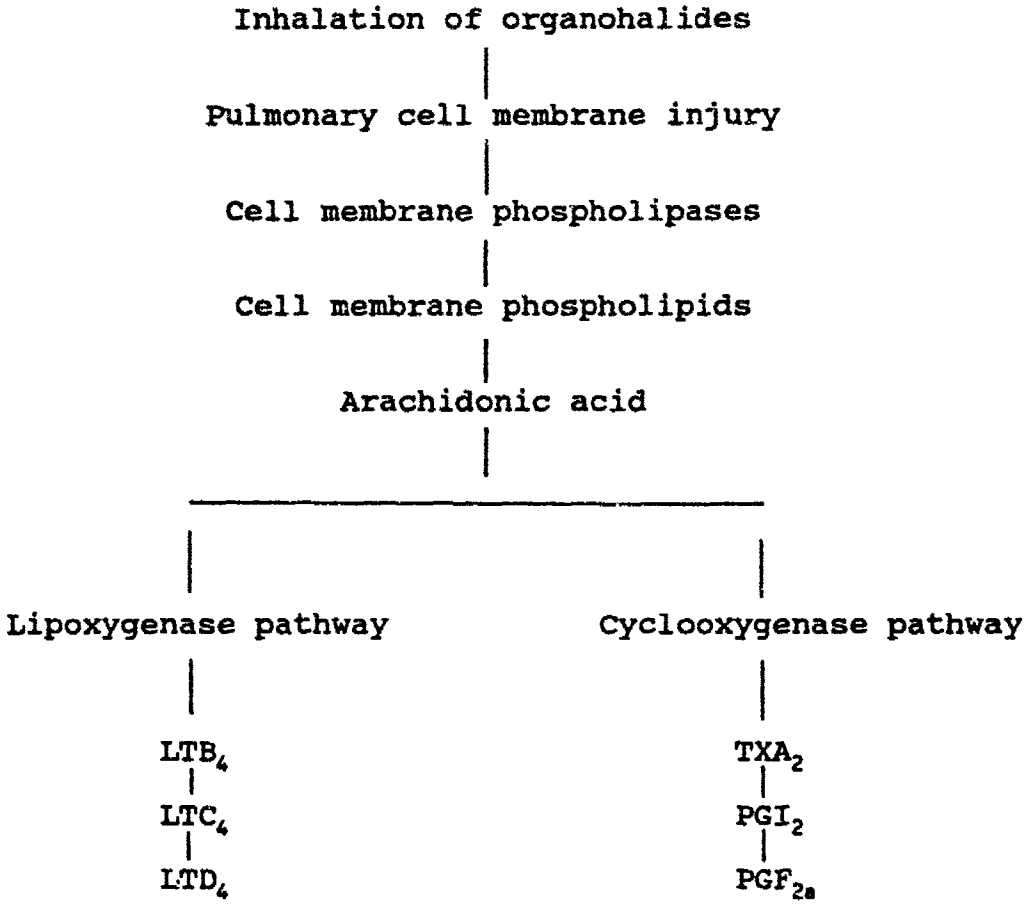
ORGANOHALIDES (OH)

- Industrial waste products
- Inhalation of OH can induce acute lung injury and pulmonary edema
- Three OH compounds that are currently of interest to the medical chemical defense community:
  - PFIB
  - TFD
  - Phosgene
- The mechanism of action of these compounds is still not fully understood.

PROPOSED MECHANISM OF ACTION OF OH

- Pulmonary vascular and bronchial constrictors:
  - Thromboxane
  - Prostaglandins
  - Leukotrienes
- Increasing pulmonary microvascular permeability:
  - Leukotrienes

PROPOSED MECHANISM OF ACTION

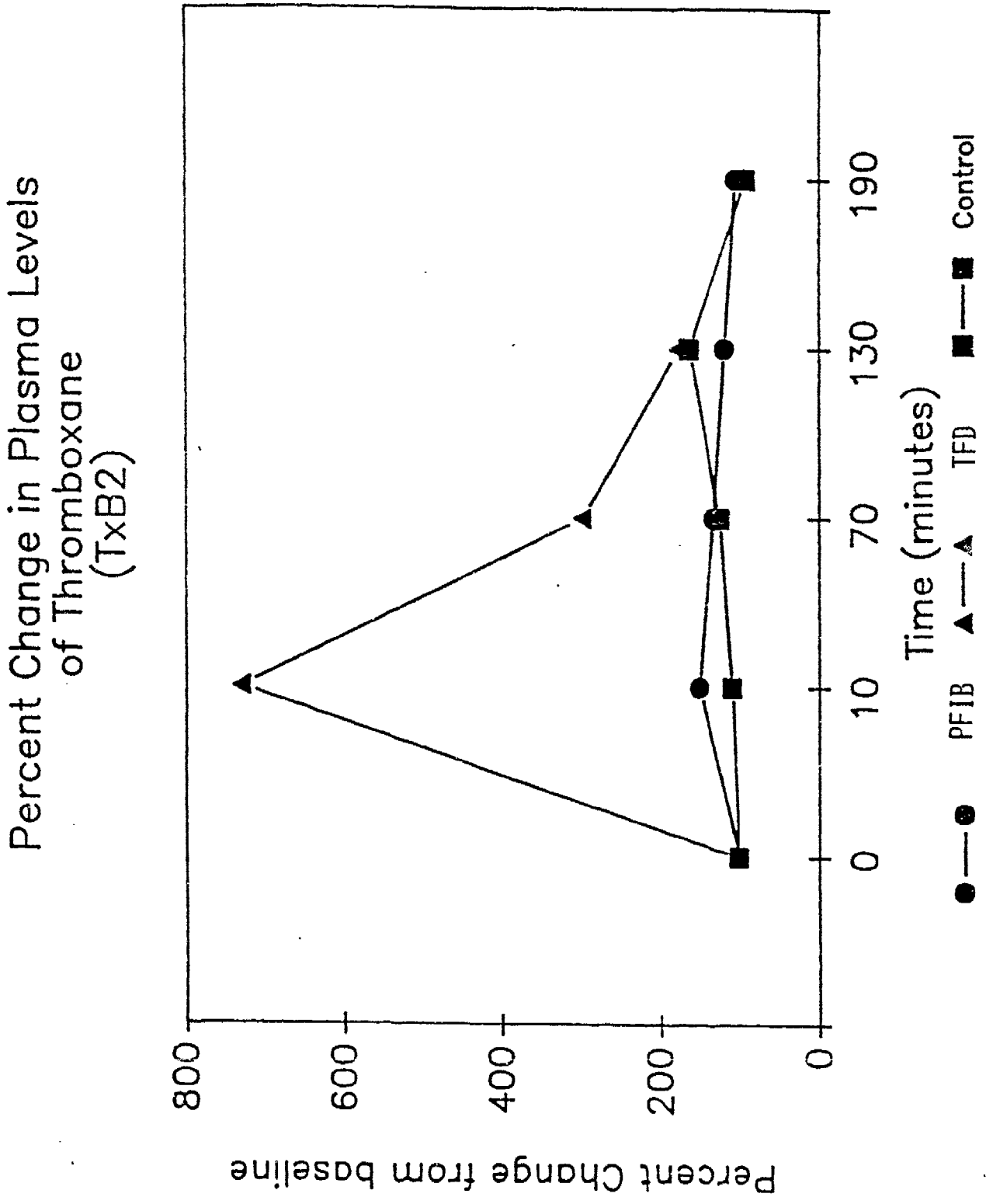


EXPERIMENTAL DESIGN

- Unanesthetized sheep were exposed for 10 minutes either to toxic doses of:
  - PFIB
  - TFD
  - Room air
- Blood samples were collected at different stages of the experiment.
- Plasma samples were assayed by RIA for the most stable metabolite of prostacyclin (6-Keto-PGF_{1a}), and thromboxane (TXB_2).
- All values were considered as % change from the pre-exposure value.

OUR PRELIMINARY DATA SUGGEST THAT:

- Arachidonic acid metabolites may be involved in the early stage of acute lung injury induced by TFD, and their release may be partially responsible for the pathophysiological changes associated with exposure to that compound.
- Specific inhibitors to enzymes in the arachidonic acid cascade may be of therapeutic value in attenuating clinical symptoms of pulmonary toxicity associated with OH exposure.
- PFIB and TFD may act by two different mechanisms in inducing acute lung injury and pulmonary edema.
- It appears that TFD acts by injuring pulmonary endothelial cell membrane. This observation, as well as alveolar epithelium injury, was confirmed by ultrastructural study.


Percent Change in Plasma Levels
of Thromboxane
(TxB2)
Percent Change from baseline
800
600
400
200
0
0
10
70
130
190
Time (minutes)
PFIB
TFD
Control

Percent Change in Plasma Levels
of Prostacyclin
(PGI)
Percent Change from baseline
800
600
400
200
0
0
10
70
130
190
Time (minutes)
PFIB
TFD
Control

WORK IN PROGRESS

- USAMRICD Protocol # 1-03-89-000-A-516
- Title: Biochemical Markers of Chemical Warfare Agent-Induced Pulmonary Edema and Acute Lung Injury.
- Proposed experimental design:
  - PFIB
    10 Sheep
  - TFD
    10 Sheep
  - Phosgene
    10 Sheep
  - Control
    10 Sheep
- Proposed studies:
  - Prostaglandins, Lung peptides, Leukotrienes — lung tissue, plasma, BAL
  - Neutrophil kinetics
  - Chemotactic factors and complement fragments — BAL
  - Histopathology and electron microscopy — lung tissue
- Collaborations:

  Dr. Sami Said, University of Illinois,
  College of Medicine at Chicago

  Dr. James Roth, Iowa State University
  College of Veterinary Medicine

DISCUSSION AFTER DR. ASSAAD'S PRESENTATION

Dr. Fowler began by asking if Dr. Assaad performed BAL (bronchiolar alveolar lavage). In response Dr. Assaad explained that this was done after intubating one lobe of the lung after killing the animal; he clarified that they did not perform tracheal intubation. Dr. Glauser asked how they explained the observation that there were no changes in hemodynamics following release of the factors discussed. That is, how could there be a release of these vasoactive substances without a change in hemodynamics? Dr. Assaad responded that, due to the complex exposure system and the timing of the exposures themselves, it was not possible to carefully perform hemodynamic measurements.

Dr. Said noted that the action of PFIB was not associated with mediators, whereas mediators were associated with the effects of TFD. Both compounds, however, produced lesions, i.e., acute lung injury. Dr. Assaad replied that it is likely that there are multiple mechanisms of action. First, with TFD, there may have been injury to the cell skeleton itself. With PFIB, at least two possibilities exist. First, PFIB may induce a lesion in the "surfactant system," with an end result of fluid entry into the alveolar space. This, of course, would be similar to the action of phosgene. Alternatively, there may have been nonselective contraction of endothelial cells and, although this is a rare mechanism, it would result in pulmonary edema. Concluding this aspect of the discussion, Dr. Said noted that it was a milestone that scientists understood that these mediators are not simply a nonspecific indicator of injury, but that the arachidonic acid products are selective to the type of injury. Dr. Assaad concurred.

Dr. Carchman asked whether Dr. Assaad had considered the roles of calcium and of changes in intracellular pH in the biochemical pathways of the phospholipase system. He clarified this comment, saying that the use of calcium probes may help identify cell targets that are indicative of the origin of cellular damage in this system. Dr. Assaad agreed that it is true that calcium is indeed involved in cell death and activation of the cascade of arachidonic acid metabolites. Dr. Assaad noted, however, that his data concurred with those of Dr. Petrali which suggested that the initial site of injury was the endothelium.

GENERAL DISCUSSION

This session began with MAJ Moore discussing the role of granulocytes in organohalide-induced lung injury. He noted that, in conjunction with Dr. Hurt, his laboratory had performed studies which indicated that peripheral white blood cell counts measured during and after exposure to all three compounds (i.e., PFIB, TFD, phosgene) decreased dramatically, and that these values returned to normal within 10-15 minutes after exposure. This response was not related to hemoconcentration or to hemodilution. Dr. Fowler noted that a precipitous fall in neutrophils is a hallmark event in nearly all forms of lung injury. He went on to explain that activities which occur in the alveolar space can influence events that occur in the vascular space. For example, the secretions of chemoattractants (whether they be products of arachidonic acid metabolism or the secretion of complement products) will enter the alveolar space after being secreted into the vascular space. It is likely these secretory products and metabolites are capable of controlling adhesion and migration from the vascular space to the lungs. It is postulated that it is this transition (i.e., the movement across the space) which itself may induce the primary cell injury to the lung tissues.

Dr. Said said that, since the pictures shown by MAJ Moore did not indicate granulocytes in the lungs, it was unclear to him just where those white blood cells went. Dr. Fowler reminded everyone that the lungs were very effective filters; thus, he suggested, the lungs would be the likely location. MAJ Moore indicated that, due to the design of the experiments, it was simply not known if the precipitous fall of leukocytes was truly associated with a change in leukocyte levels in the lung. He explained that it had been necessary to transport animals from one location to another during the course of these experiments, and that the lung histology was performed 4-5 hours after exposure, which was long after the leukocytes had returned to normal. Thus, it simply could not be determined where the cells went during the period when their levels were very low. In addition, some of these observations were made in rats and other observations in sheep; as a result, MAJ Moore noted, they did not have a complete set of data for either species (i.e., serial histopathology matched to the temporal changes in leukocyte levels).

Dr. Assaad made the point that, in general, granulocytes are less flexible (i.e., less deformable) than red blood cells, and it is difficult for them to squeeze through small spaces. Consequently, it is possible that as a result of platelet "bridging," the neutrophils may essentially get stuck in their movement

between tissues. (However, Dr. Fowler commented that the rigidity of the granulocyte membrane varies, being a function of overall metabolic activity and the state of activation. For example, membranes are rigid only when receptors are being activated; at other times they are reasonably flexible.)

Finally, Dr. Petrali noted that in the EM studies, there was marked vascular congestion at 5 minutes. He added that granulocytes were the primary type of blood cell observed.

EXERCISE POTENTIATION OF EXPRESSION OF LUNG INJURY INDUCED BY PFIB AND TFD

Dr. Bruce E. Lehnert

A study to examine exercise potentiation of expression of lung injury induced by PFIB and TFD is being performed at the Los Alamos National Laboratory. The objectives of the study are: (1) to characterize the potentiation of expression of lung injury induced by PFIB and TFD using lung gravimetric and histopathologic criteria; (2) to characterize the post-exposure period of time during which the expression of PFIB- and TFD-induced lung injury can be potentiated by exercise, i.e., the "window of susceptibility"; (3) to determine whether two exercise bouts performed during the window of susceptibility can cause even more marked increases in the expression of lung injury; and, if so, (4) to determine whether such bouts are additive or synergistic in enhancing PFIB- and TFD-induced lung abnormalities; and (5) to determine if early post-exposure exercise extends the post-exposure window of time within which exercise can potentiate the expression of lung injury caused by inhaling PFIB and TFD. This investigation essentially follows the experimental design used in recent assessments of the effects of post-exposure exercise on the expression of lung injury induced by nitrogen dioxide (NO_2) [Environ. Res. 48:87-99, 1989]. In this presentation, an overview of the general design and observations made in the NO_2 study is provided, and associated protocols used in that study are described which will be utilized in the PFIB and TFD investigations. Additionally, a system is described for exposing laboratory rats to PFIB and TFD, and approaches for elucidating biophysical and cellular mechanisms involved in acute lung injury and permeability pulmonary edema are discussed.

To date, no actual data have been obtained with PFIB and TFD.

EXERCISE POTENTIATION OF EXPRESSION OF LUNG INJURY INDUCED BY PERFLUOROISOBUTYLENE AND BIS(TRIFLUOROMETHYL)DISULFIDE

Dr. Bruce E. Lehnert and Douglas M. Stavert

INTRODUCTION

In April 1989, a study was initiated at the Los Alamos National Laboratory (LANL) to examine exercise as a potentiator of lung injury induced by acute exposures to perfluoroisobutylene (PFIB) and bis(trifluoromethyl)disulfide (TFD). The specific objectives of this project are: (1) to characterize the potentiation of expression of lung injury induced by PFIB and TFD using lung gravimetric and histopathologic criteria; (2) to characterize the post-exposure period of time during which the expression of PFIB- and TFD-induced lung injury can be potentiated by exercise, i.e., the "window of susceptibility;" (3) to determine whether two exercise bouts performed during the window of susceptibility can cause even more marked increase in the expression of lung injury; and, if so, (4) to determine whether such exercise bouts are additive or synergistic in enhancing PFIB- and TFD-induced lung abnormalities; and (5) to determine if early post-exposure exercise extends the post-exposure window of time within which exercise can potentiate the expression of lung injury caused by inhaling PFIB and TFD. Inasmuch as this study essentially follows the experimental design of our recent assessments of the effects of post-exposure exercise on the expression of nitrogen dioxide (NO_2)-induced injury [1], we summarize in this report some findings made in our previous study in order to illustrate how post-exposure exercise can augment the severity of pulmonary injury in previously damaged lungs. In addition, we describe: (1) protocols that we use in our studies of post-exposure exercise effects, (2) an exposure system developed at LANL to administer PFIB and TFD to laboratory rats, and (3) new approaches developed at LANL to elucidate biophysical and cellular mechanisms that underlie acute lung injury and permeability pulmonary edema.

NO_2 Injury and Potentiation of Expression of NO_2 Injury by Post-Exposure Exercise (E)

In our NO_2 study, rats were exposed [2] to 100 ppm NO_2 or filtered air only for 15 min; previous investigations in our laboratory revealed that this NO_2 exposure concentration and time produced histopathologic and lung gravimetric changes as of 1 hr post-exposure that were consistent with acute lung injury and permeability pulmonary edema. The rats were then subdivided into groups to be sacrificed at 1, ~8, or ~24 hr after exposure. The

general approaches used for NO_2 and E studies, which will also be used for the PFIB and TFD investigations, are as follows: To determine the immediate effect E has on NO_2-induced pulmonary injury, some groups of rats were exercised on a treadmill 1 hr before sacrifice. To characterize the course of progression of E-associated potentiation of NO_2-induced injury, some rats were exercised immediately after NO_2 exposure and their lungs were examined 8 hr later. Still other groups of NO_2-exposed animals were exercised 8 or 24 hr after exposure and sacrificed at 24 hr, or ~8.5 hr and 24.5 hr, respectively, after exposure in order to gain information on the potentiating effects of E when performed at latter post-exposure times. To elucidate potential additive or synergistic effects of two post-exposure E bouts, some animals that were exercised immediately after the NO_2 exposure were again exercised at 8 hr and sacrificed either after the second run (~8.5 hr) or 24 hr later. To determine if early post-exposure E could extend the window of time within which E potentiates injury, some rats that were exercised immediately after NO_2 exposure were again exercised 24 hr post-exposure and subsequently sacrificed. Air-exposed controls were either rested after exposure or exercised and sacrificed 1, 8, or 24 hr after exposure.

By a variety of semiquantitative histopathologic criteria, the performance of E after NO_2 inhalation was found to substantially increase the severity of expression of pulmonary edema (Figure 1). Although specific details on the histopathologic observations made following each post-NO_2 regime outlined above are beyond the scope of this report [1], it is interesting to note that E did not appear to result in an increase in Type II cell hyperplasia. Such evidence suggests that Type I pneumocyte injury was not increased by E, and accordingly, further suggests that the increase in severity of expression of lung injury by E may not have been due to an actual increase in the severity of the underlying initial lesion induced by NO_2. For the sake of brevity, we present here an overview of our observations of the effects of E on NO_2-induced lung injury using alterations of lung wet weights (LWW) to index the extent of pulmonary edema (Figure 2); lung dry weight changes, which generally paralleled LWW changes, are summarized elsewhere [1]. A summary of the LWW data in Figure 2 is as follows.

- Sham Air Exposure and E: Post-exposure exercise does not lead to an increase in LWW of rats exposed to air only.

- NO_2 Exposure and E Immediately Thereafter: The LWW of rats exposed to NO_2, exercised immediately thereafter, and sacrificed 30 min later were ~40% greater than the LWW of animals that were exposed to NO_2 but rested prior to sacrifice. The E-associated increases in LWW were persistent. For those

animals exercised immediately after exposure and sacrificed 8 hr thereafter, their LWW were elevated ~30% above their equivalently exposed but rested counterparts. As of 24 hr after immediate post-exposure E, the LWW were increased ~45% above the LWW of NO_2-exposed but rested animals.

- NO_2 Exposure and Exercise at 8 or 24 hr Post-Exposure: When animals were exercised 8 hr post-exposure and sacrificed shortly thereafter, their LWW increased to levels comparable to those found at the 8-hr sacrifice time point following NO_2 exposure and immediately exercised. When the rats were exercised 8 hr post-exposure and examined 16 hr later, their LWW values were similar to those obtained with rats exposed to the NO_2 and allowed to rest prior to sacrifice 24 hr after exposure. Thus, the E-associated increases in the LWW of rats exercised 8 hr post-NO_2 exposure and sacrificed shortly thereafter subsided by the 24-hr sacrifice time.

  E performed 24 hr after NO_2 exposure did not significantly increase LWW. Thus, the window of susceptibility to the magnitude of NO_2 injury produced in this study was closed as of 24 hr after exposure.

- NO_2 Exposure and Immediate and Subsequent Exercise: The average LWW of rats exercised 8 hr following immediate post-exposure E increased ~25% above that observed at the 8-hr post-exposure time with rats exposed to the NO_2 and exercised immediately thereafter. No evidence for a persisting two-exercise-bout-associated increase in LWW, however, was found when rats were exposed to the NO_2, exercised immediately and 8 hr thereafter, and allowed to rest until sacrifice 24 hr after exposure. The LWW of animals exercised immediately and 24 hr after exposure appeared to be higher than the LWW of rats that were only exercised immediately after the NO_2 exposure and sacrificed at 24 hr, suggesting that exercise shortly after NO_2 exposure can extend the window of susceptibility.

The mechanisms involved in enhancements in the expression of pulmonary edema following the inhalation of a toxic agent such as NO_2 have not been delineated. Aside from the effects E may have on cellular changes in NO_2-exposed lungs, the potentiated expression of injury may be due largely to physical mechanisms. Several investigators [3-6] have found an apparent uncoupling between diffusion capacity and cardiac output after E that is consistent with an E-associated, transient, subclinical pulmonary edema. In the context of Starling's equation for fluid movement, when normal lung permeability is breached and pulmonary edema ensues, E-associated elevations in cardiac output and pulmonary

vascular pressure would be expected to facilitate the translocation of water and blood solute constituents into extravascular compartments. Accordingly, the potentiation in expression of NO_2-induced lung injury by E may represent the imposition of forces involved in bringing about quasi-hydrodynamic pulmonary edema atop the complex factors involved in permeability pulmonary edema. Another factor possibly underlying the potentiation of expression of lung injury is the increase in ventilation associated with E. Albelda and colleagues [7] have shown that when ventilation was increased in sheep, lung lymph flow increased and the ratio of total protein concentration in the lymph relative to plasma fell. Such results are consistent with a hyperpnea-related increase in the pulmonary transvascular hydrostatic pressure gradient. Of importance, pulmonary lymph flow has also been reported in increase (up to threefold) in exercising sheep.

Although physiologic considerations may offer some explanation for various aspects of the potentiation of expression of NO_2-induced pulmonary edema, they do not provide direct insight as to the underlying bases of others. For example, it is unclear why the kinetics of subsidence of pulmonary edema following immediate post-exposure E and immediate and 8-hr post-exposure E differ. Our findings seem to point to the translocation of water and blood solutes into at least two compartments that have differing rates of resolution of edema fluid. Immediate post-NO_2 exposure E appears to result in the further filling of one of these with edema fluid beyond the level produced by NO_2 exposure and rest only. This potential compartment evidently continues to fill for at least 24 hr post-exposure. On the other hand, a second, more rapidly resolving compartment accumulates fluid during or shortly after a second E bout following the first. It is tempting to speculate that the immediate post-NO_2 exposure E bout resulted primarily in the further filling of the lung's interstitium with fluid, whereas the second E bout mainly promoted the translocation of edema fluid from the already well-filled interstitium into the more rapidly clearing alveoli [8]. From a mechanistic perspective, the driving force(s) favoring such fluid movements could include E-associated increases in vascular distension with corresponding decreases in interstitial compliance, increased vascular filtration pressure, and an already well-hydrated interstitial compartment.

Exercise Protocols and Techniques to be Used in the PFIB and TFD Studies

Before use, animals will complete a 19-day training program designed to behaviorally and physically condition them to perform on a treadmill [9]. The training program is initiated on the first day of training by running rats on a treadmill at low work

loads (10-15 m/min, 15% grade) for five intermittent periods. Each of these training bouts is conducted for a duration of 2 min with 2-min rest periods between each running episode. The work intensities and durations of exercise are increased daily until the rats run 40 m/min, 4 times per day as of day 13. From day 13 through day 19, the rats exercise twice per day using a "ramp" exercise protocol (to be described). As illustrated in Figure 3, the VO_{2MAX} values of animals performing the ramp protocol become satisfactorily stable and reproducible during the last several days of the training program.

Prior to the ramp exercise runs, rats are subjected to a "familiarization run" consisting of two periods of short-duration exercise (20 m/min for 3 min, 15% grade) separated by a 3-min rest period and finally followed by a 10-min rest period before initiation of the actual ramp protocol. The ramp run is begun by running rats at an initial treadmill velocity of 10 m/min. Every 30 sec thereafter, the treadmill speed is incrementally increased by 5 m/min while the animals run at a constant 15% grade (Figure 4). Maintenance of running pace is encouraged by electro-stimulation delivered via a grid mounted behind the treadmill. The treadmill used in these studies is contained in a metabolic chamber (Figures 5 and 6). In the PFIB and TFD studies, VO_{2max} during post-exposure exercise will be measured to index decreases in work performance capacity of exposed animals. Preliminary data obtained in our NO_2 studies suggest that reductions in work performance capacity may directly scale with the extent of pulmonary edema (Figure 7).

Animal Exposures to PFIB and TFD

Exposure Methods: All operations for storage of the material, atmosphere generation, animal exposure, and atmosphere-fusing are conducted within a Labconco Carcinogen/Toxic Gas Glove Box. This glove box has internal HEPA filters on the inlet and exhaust as well as activated charcoal filters (3.5 lb) on the exhaust. An internal blower maintains a glove box airflow rate of 70 cfm.

Atmosphere Generation: Exposure atmospheres are contained within an exposure system that includes an atmosphere generator, delivery tubing and valves, an animal-exposure tube and a charcoal-absorbing bed (Figure 8). Exposure atmospheres up to 100 mg/m^3 (~12 ppm) will be generated for up to 4-hr periods of time. The atmosphere generator is slightly different for PFIB and TFD. PFIB is contained within its storage cylinder at a pressure of ~200 mm Hg (4 psig). A normally closed solenoid valve and an SS micrometering valve are attached to the neat agent canister. Microbore tubing connects the canister with a 5-ml gas-tight syringe via a three-way valve. The micrometering

function and microbore tubing resistance allow for controlled filling of the syringe before every exposure. The three-way valve is switched to allow neat agent to be pumped into the dilution-air-mixing manifold. Dilution air is piped into the glove box from an external oil-less air compressor and metered via a flow-controller to provide a constant 2 liter/min airflow rate through the exposure system. Exposure atmosphere generation begins with the starting of the syringe pump, which delivers up to 0.024 ml/min of neat agent into the dilution airstream to produce the maximal exposure concentration of 100 mg/m^3. TFD is handled in an identical way except that the neat material canister is warmed to 37°C to generate enough vapor pressure within the canister to allow material to exit from the canister. Downstream from the mixing manifold, a mixing chamber assures complete mixing before the desired atmosphere is delivered to the animal. The exposure airstream is then passed through a valve (V1) before delivery to the animal exposure tube. V1 is a large-bore Teflon three-way valve, and when actuated with another valve (V2), it provides a means for the exposure atmosphere to bypass the animal-exposure tube while simultaneously delivering clean air to the animal. This allows for the safe installation and removal of the animal-exposure tube as described later. V2 is a flow-control valve with solenoid ON-OFF features. This valve is operated from outside the glove box and is connected to the KIVA clean compressed air. Downstream from the animal-exposure tube and bypass circuit, a charcoal absorption tower captures the exposure atmosphere and allows for the slow release of the compounds into the glove box. The exposure system is designed to work at atmospheric pressure or slightly positive pressure (<0.1 cm H_2O) relative to the glove box but is incapable of overpressurization. Measurement of the exposure atmosphere by gas chromatography occurs from ports located prior to the animal-holding tube, after the animal-holding tube, and at the exhaust of the charcoal filter stack. Real-time measurements of exposure concentrations are performed on a continual basis using a Miran analyzer (infrared). The glove box interior is also monitored via gas chromatography.

A compact, dedicated, dual-column gas chromatograph has been built for monitoring halogenated compounds in air. The detector for the GC peaks is an electron capture detector (Model 140B, Valco Instruments) for maximum sensitivity. The carrier gas is argon/methane (90:10), passed through a heated catalytic purifier (Supelco, Inc.) to remove oxygen and water. Column and sample-loop purge flows (10-20 cm^3/min) are controlled by pressure regulators and needle valves. Sample loops and chromatographic columns (0.5 and 1 mm x 3 mm o.d. Teflon columns containing 80/100 mesh Chromosil 310 from Supelco, Inc.) are contained within a heated (70°C) valve oven (Carle Instruments, Model 4300) and attached directly to electrically actuated valves. Output

from the electron capture amplifier is sent to a chart recorder/ chromatographic peak integrator (Spectra Physics SP 4270).

A valve sequence programmer (Valco Instruments) and solenoid valves are used to automatically initiate sampling and analysis at periodic intervals (2-10 min), while venting the interfering peak of oxygen. Air is drawn continuously through sampling loops except when being dumped briefly to the GC columns. Careful adjustment of column lengths, carrier flows, and temperature can allow overlapping chromatograms from simultaneous sampling at two points.

This apparatus is calibrated using perfluoro-2-butene, a much less toxic isomer of the compound of interest. Sensitivities should be equal and GC retention times should be similar to those of PFIB and TFD. Serial dilutions using syringes provide orders-of-magnitude calibrations to the most sensitive range. A detection limit of <1 ppb has been determined.

Animal Exposures: Animals are exposed to the atmospheres in Teflon animal-exposure tubes for periods of 10 min. Up to 10 animals per day will be exposed in a sequential fashion. Once the atmosphere has been set up and diverted into the exposure atmosphere bypass circuit, the animal is sealed into the animal-exposure tube and passed into the glove box through its airlock. The animal-exposure tube is secured into the exposure system with swagelock type fittings. The atmosphere is then directed to the animal-holding tube for exposure. After the exposure, fresh air is directed to the animal-exposure tube for a minimum period of 10 min or until sampling via the gas chromatograph and Miran analyzer indicates no detectable atmosphere contamination within the animal exposure tube. At this time, the animal-exposure tube is disconnected from the exposure system and passed into the laboratory area via the airlock.

A minimum of two exposure concentrations of PFIB and TFD are to be examined in the exercise studies. Preliminary studies are being directed to assessments of the magnitude of lung injury caused by exposing the animals to PFIB and TFD over a range of mass concentrations. Mass concentrations to be evaluated for PFIB will initially be 50 mg/m^3 and 5 mg/m^3. Based on the level of injury observed in the lungs of animals exposed to these concentrations, a third mass concentration within the 50 to 5 mg/m^3 range, e.g., 10 mg/m^3, will also be evaluated. TFD will be initially evaluated at mass concentrations of 70 mg/m^3 and 7 mg/m^3, with a third mass concentration within this range being selected and tested as for PFIB. The level of pulmonary injury in the above studies will be assessed as of 24 hr after exposure. The goals of this effort are: (1) to identify mass concentrations of PFIB and TFD that cause no or trivial lung

injury under resting conditions, and (2) to identify mass concentrations of PFIB and TFD that produce significant but sublethal injury.

Histopathology

In addition to using lung wet and dry gravimetric criteria, lung injury and pulmonary edema following PFIB and TFD exposure will be characterized histopathologically. The trachea and lungs of exposed rats will be fixed by submersion in 10% formalin phosphate buffered saline for 48 hr. After fixation, the left lung lobe will be sliced uniformly from the apex to the base along a line from the most posterior to the most anterior aspect in order to expose the maximal planar surface area for sectioning. The blocks of tissue are embedded in paraffin, and 4-μm sections are prepared and stained with hematoxylin and eosin by conventional methods. Histopathologic analyses will focus on alterations in the periterminal brochiolar-alveolar duct-alveolar region, i.e., abnormal accumulations of edema fluid and fibrin, accumulations of polymorphonuclear leukocytes and alveolar macrophages in the alveolar spaces, the extravasation of erythrocytes in the alveolar spaces, vascular congestion, alveolar septal edema, thickening of the interstitial space, and alveolar cuboidal cell hyperplasia, i.e., type II cell hyperplasia. A grading scale will be used to quantitatively describe each of the above pathologic features for comparative purposes in terms of their: (1) distribution, i.e., relative number of periterminal bronchioles showing a lesion in associated alveolar structures, (2) severity, or the relative number of periterminal bronchiolar structures affected, and (3) intensity, i.e., the relative amount of material or relative alterations of cells in the alveoli. The distribution index scale for a given feature, e.g., fibrin accumulation, will range from 0 to 4, with 0 = not observed, 1 = single for focal in appearance, 2 = few but multifocal, 3 = moderate number to many involved terminal airways, and 4 = all or essentially all periterminal bronchiolar-alveolar structures are affected, i.e., diffuse. The relative severity index for a given pathologic feature ranges from 0 to 3, with 0 = no observable abnor-mality, 1 = the focal appearance of the abnormality in the periterminal alveolar structures, 2 = several affected alveolar structures, and 3 = many to all periterminal bronchiolar alveolar structures demonstrate the disorder with confluence of adjacent lesions. The relative intensity index ranges from 0 to 4, with 0 = no detectable abnormality, 1 = trace but detectable alterations in amount of abnormal material or numbers of cells, 2 = mild amount or small but abnormal material or number of cells, 3 = moderate amount of material or moderate numbers of cells, and 4 = large amounts of intra-alveolar material or large changes in cell numbers. For grading vascular congestion, only

the distribution and severity indices will be used. In this case, the distribution index will refer to the relative numbers of periterminal bronchioles that show capillary congestion in associated alveolar septal walls and the severity index will reference the relative numbers of the alveoli involved.

New Approaches for Elucidating Biophysical and Cellular Mechanisms of Acute Lung Injury and Pulmonary Edema

While some progress has been made in characterizing events associated with acute lung injury and the development of permeability pulmonary edema, a comprehensive understanding of mechanisms underlying these processes is lacking. Below we describe two approaches currently being used in our laboratory to gain further information about: (1) the integrity of the alveolar-capillary barrier as indexed by the passage of blood proteins into the lung's air spaces, and (2) the cellular bases of acute lung injury in the context of the development of permeability pulmonary edema.

Examination of Lavage Fluids for Blood Compartment Constituents: We have developed an HPLC system to quantitatively resolve numerous protein constituents (as well as several non-protein constituents) in the extracellular fluid lining of the lung as sampled by bronchoalveolar lavage [10] (Figure 9). Three of the fractions resolved are common to the blood compartment. Fractions 3 and 4 have been identified as transferrin and albumin, respectively, and Fraction 5 represents immunoglobulins G and M. In a preliminary study to assess the utility of the HPLC approach for assessing the translocation of blood compartment constituents into the alveoli due to a toxic insult to the lung [11], rats were intratracheally instilled with 7 μg cadmium (Cd) chloride and their lungs were lavaged 24 hr thereafter. Hallmarks of acute injury following the deposition of the heavy metal Cd in the lung include damage to Type I pneumocytes, a marked influx of polymorphonuclear leukocytes (PMN) into the alveoli, extravasation of erythrocytes into the air spaces, and alveolar flooding with proteinaceous fluid. HPLC analyses of the lavage fluids from the Cd-instilled animals revealed that Fractions 3, 4, and 5 were all equivalently increased approximately 25-fold (Figure 10). Thus, the passage of blood proteins into the alveoli following Cd treatment appeared to be independent of the molecular weights of the proteins. In a related study [11], we analyzed the lavage fluids obtained from rats 24 hr after 15-min exposures to 100 ppm NO_2 (Figure 11). In this case, Fractions 3, 4, and 5 were increased 72-fold, 48-fold, and 35-fold, respectively. The differences in the nature of the "leaks" observed following Cd- and NO_2-induced lung injury may point to fundamental differences in the effective "pore" sizes caused by these

two toxic agents. Additionally, the detected appearance of transferrin in the alveolar compartment following both Cd and NO_2 treatment, and the known role of iron in mediating lipid peroxidation, suggests that this blood protein may be involved as a secondary factor in mediating a subsequent injurious response to permeability-altering agents. Regardless, our investigations to date, which have focused on several different models of lung injury [11-13], indicate that HPLC analyses of proteins in bronchoalveolar lavage fluids provides a powerful approach for quantitating the translocation of blood compartment constituents into the lung's air spaces, as observed in permeability pulmonary edema. Moreover, the sensitivity of the HPLC method and the fact that only small quantities of material are required for the HPLC analyses further suggest that such analyses may be performed on sputum samples to detect and monitor the course of acute lung injury and pulmonary edema in the human. It should also be pointed out that HPLC analyses of protein constituents in lavage fluids that are not common to the blood compartment may provide an approach for monitoring the status of the lung's cellular populations following environmental insults.

Isolation of Specific Lung Cell Subpopulations: The lack of readily available specific lung cell phenotypes for subsequent *in vitro* analyses of cells in primary culture has limited the development of a much needed information base on cellular mechanisms involved in acute lung injury and pulmonary edema. Over the last several years, we have been developing multilaser/multiparameter flow cytometric techniques to isolate various lung cell subpopulations in high states in purity and viability for subsequent *in vitro* study. One of these approaches, which we call the "electro-optical phenotype method," is based on the notion that cells of a common phenotype have the same electronic volumes (Coulter principle), absorb light similarly, scatter light similarly, and autofluoresce similarly when excited by laser light [14]. An advantage of this approach is that it does not require the use of special stains or fluorochrome-conjugated monoclonal antibodies, which could alter the subsequent behaviors of the cells *in vitro*, to resolve a cell type of interest that is contained in a complex cell mixture. We illustrate the usefulness of the electro-optical phenotype method here in the context of lung free cells and the isolation of polymorphonuclear leukocytes (PMN) from alveolar macrophages (AM) following the intrapulmonary deposition of Cd [15]. In Figure 12A, histograms of several flow-cytometrically ascertained parameters obtained from free cells lavaged from an untreated rat are shown. In this sample, virtually all of the cellular events analyzed were AM. Accordingly, the histograms represent the flow cytometric signatures, or electro-optical phenotypic characteristics, of normal AM. In Figure 12B, the same parameters were assessed with cells

lavaged from a Cd-treated animal. Relative to normal AM, the emergence of new peaks in the electronic volume and uv-excited blue autofluorescence parameters is especially obvious. Bivariate plots of the blue autofluorescence and electronic volume signals revealed that these parameters could be used to separately resolve the PMN from the AM (Figure 13). Sorts of the cells in the PMN window outlined in Figure 13 were found to be >95% pure PMN (Figure 14). Other cell types resolved and isolated to date in our laboratory by the electro-optical phenotype method and related flow cytometric techniques include subpopulations of AM, interstitial macrophages, mast cells, eosinophils, Type II epithelial cells, lymphocytes, and lymph nodal mononuclear phagocytes [14-21]. The availability of these and other specific lung cell subpopulations, e.g., resident Type I pneumocytes, endothelial cells, etc., offers in the future the possibility of examining each lung cell type separately following a toxic insult to the lung and will provide a means to study cellular responses of numerous lung cell types to toxic agents *in vitro*. As well, we envision that these new cell isolation techniques will provide us with the *in vitro* capability of investigating interactions of specific lung cell types that may be involved both in lung injury and in lung repair.

Summary and Relevance of the PFIB and TFD Studies at LANL

The current investigations of PFIB and TFD at Los Alamos will be completed as of April 1990. The anticipated outcomes of our studies are: (1) the development of a data base as to the expected severities of respiratory tract abnormalities that can result from acute inhalation exposures to PFIB and TFD, (2) determinations of degradations in work performance capacity following PFIB and TFD exposure, (3) the identification and characterization of exercise as a post-exposure risk factor that can augment the expression of PFIB- and TFD-induced lung injury, (4) the obtainment of pathophysiologic information that can usefully serve as a source of guidance for the pursuit of mechanisms underlying PFIB- and TFD-induced lung injury and for the rational development of prophylactic-therapeutic strategies against these agents, and (5) the availability of small-model systems for evaluating the efficacy of prophylactic and therapeutic strategies against inhaled PFIB and TFD.

Figure Legends

Figure 1: The micrograph on the left was obtained from the lung of a rat 24 hr after a 15-min exposure to NO_2. The micrograph on the right was obtained from the lung of a rat that was equivalently exposed to NO_2, exercised immediately after exposure, and sacrificed 24 hr after exposure. Exercise caused marked increases in the appearance of fibrin and extravasated erythrocytes.

Figure 2: Lung wet weight of the animals in the various exposure groups. □: air-exposed rats; ■: air-exposed and exercised rats; ●: NO_2-exposed and rested rats (associated dotted lines and arrows indicate post-exposure values obtained when animals were exercised and/or sacrificed after an exercise bout); ○: NO_2-exposed and immediately exercised animals (associated dotted lines and arrows indicate post-exposure values obtained when animals were subjected to a second exercise bout and/or sacrificed following the second exercise bout). Values in the figure represent means ± S.E. of measurements made of lungs on 4-8 rats per group.

Figure 3: During the last several days of training, VO_{2max} values of rats become relatively stable at a level that is 10-20% greater than that of unconditioned animals.

Figure 4: Illustration of the "ramp" exercised protocol showing incremental increases in treadmill velocities.

Figure 5: Photograph of a rat running on the treadmill that is contained in a metabolic chamber.

Figure 6: Schematic representation of the treadmill-metabolic chamber system used to measure VO_{2max} as an index of endurance.

Figure 7: Relationship of VO_{2max} and lung wet weights. The mean values shown were obtained from different groups of rats equivalently exposed to NO_2.

Figure 8: Schematic representation of the exposure system and associated components.

Figure 9: Resolution of 11 constituents in lavage fluid obtained from an untreated rat. Nine of the eleven fractions contain protein and three of these are common to rat blood. The other protein constituents appear to be unique to the lung.

Figure 10: Changes in the relative abundance of the various lavage constituents following the intrapulmonary deposition of Cd.

Figure 11: Changes in the relative abundance of the various lavage constituents following exposure to NO_2.

Figure 12A and B: Flow cytometric histograms of normal alveolar macrophages (A) and lung free cells following the deposition of Cd in the lung (B).

Figure 13: Bivariate combination of electronic volume and blue autofluorescence that resolves polymorphonuclear leukocytes (PMN) from alveolar macrophages (AM).

Figure 14: Micrograph of cells sorted from the PMN window outlined in Figure 13.

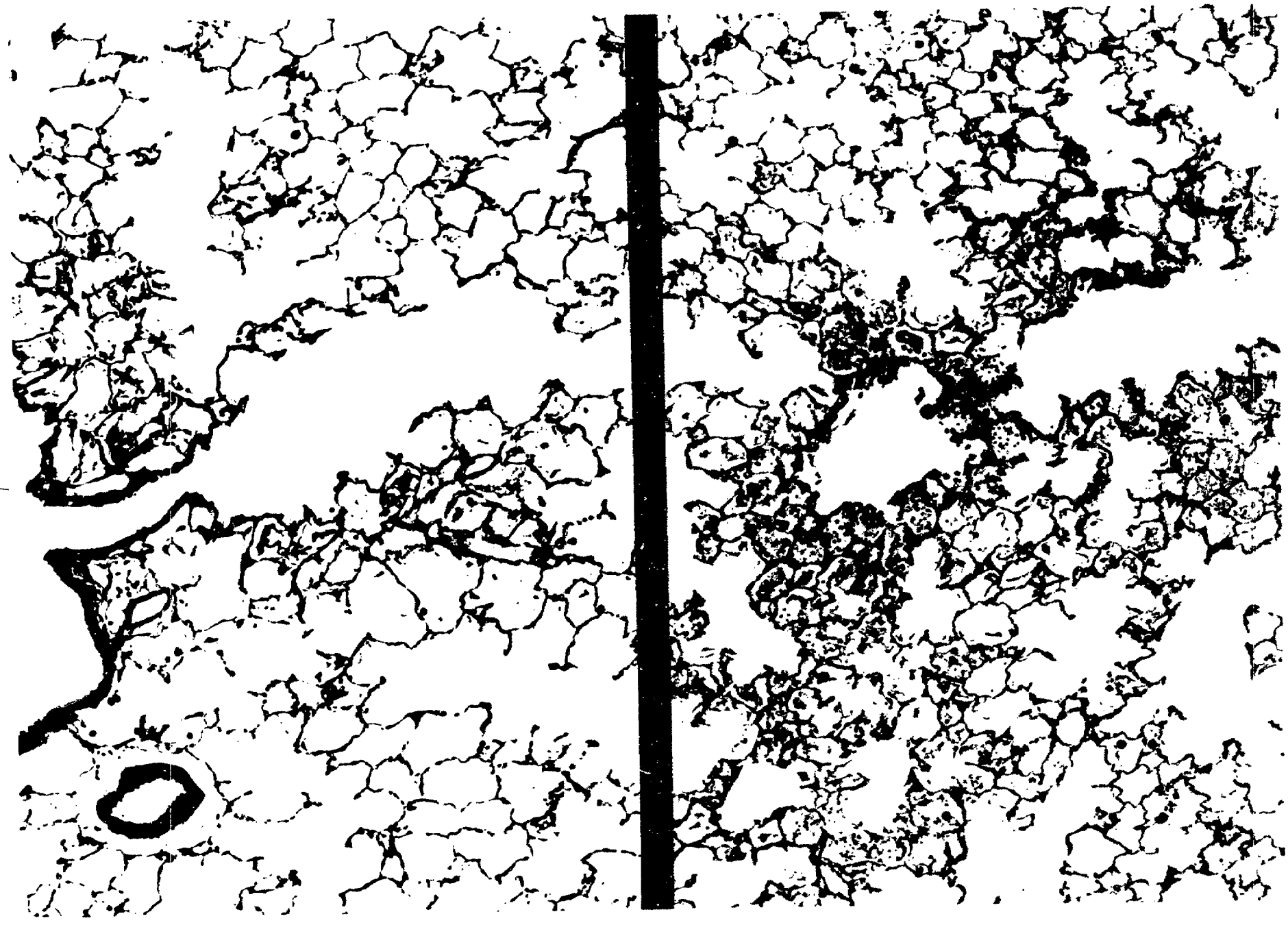


Figure 1

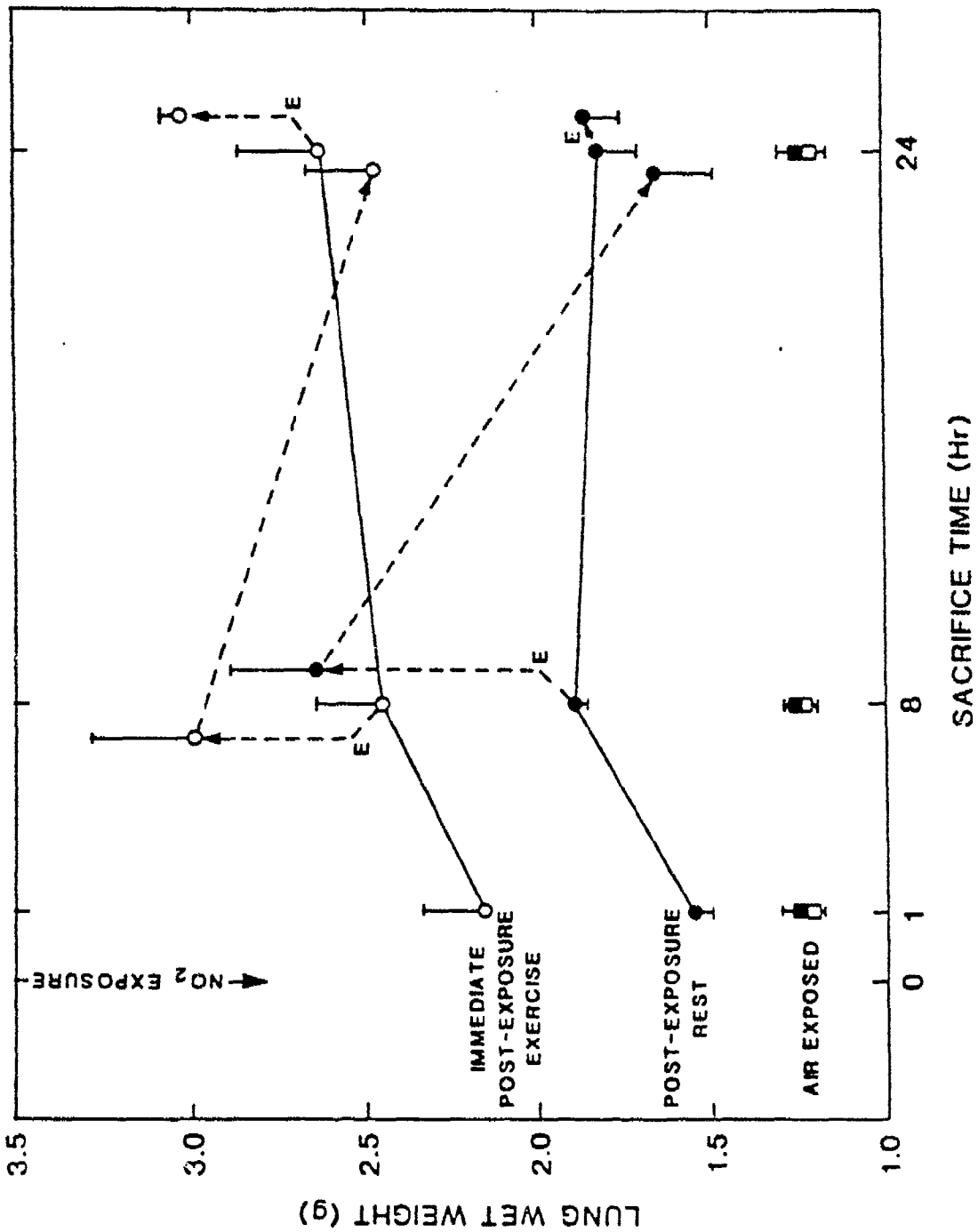

LUNG WET WEIGHT (g)
3.5
3.0
2.5
2.0
1.5
1.0
0
1
8
24
SACRIFICE TIME (Hr)
AIR EXPOSED
POST-EXPOSURE REST
IMMEDIATE POST-EXPOSURE EXERCISE
NO2 EXPOSURE
E

Figure 2

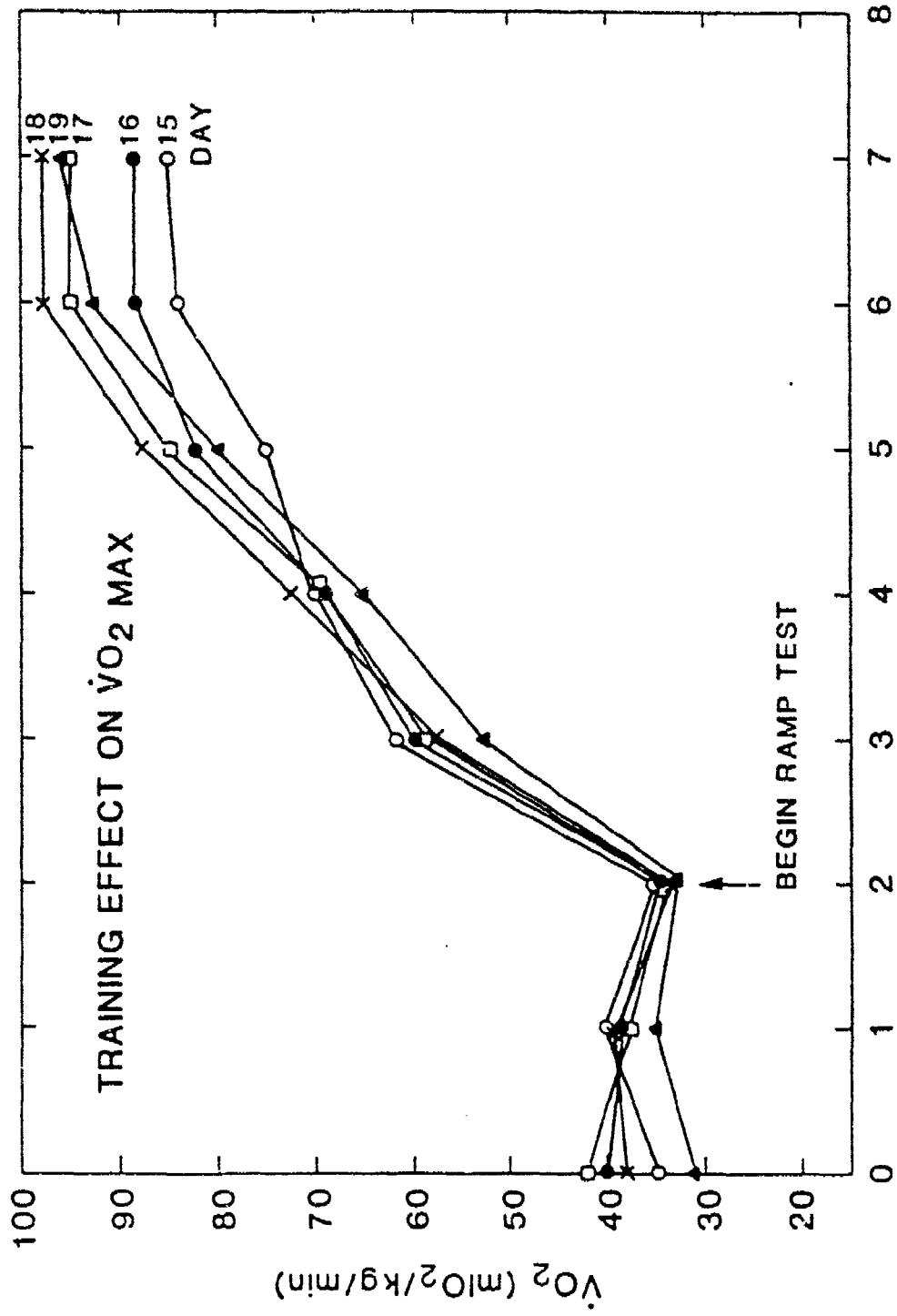

TRAINING EFFECT ON $\dot{V}O_2$ MAX
DAY
18
19
17
16
15
BEGIN RAMP TEST
TIME (min)
$\dot{V}O_2$ (mlO_2/kg/min)
100
90
80
70
60
50
40
30
20
0
1
2
3
4
5
6
7
8

Figure 3

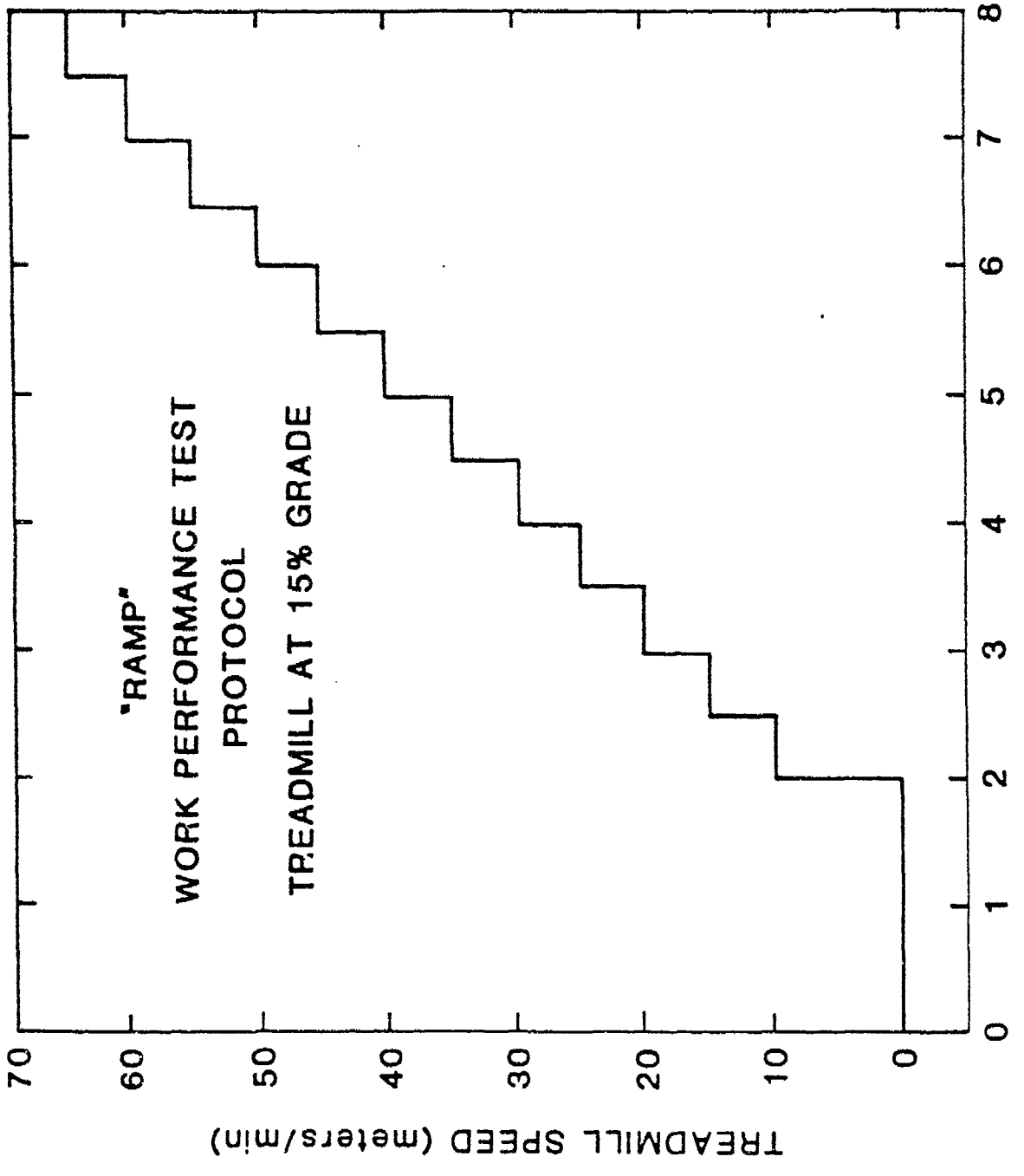

"RAMP"
WORK PERFORMANCE TEST
PROTOCOL
TREADMILL AT 15% GRADE
TREADMILL SPEED (meters/min)
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
TIME (min)

Figure 4

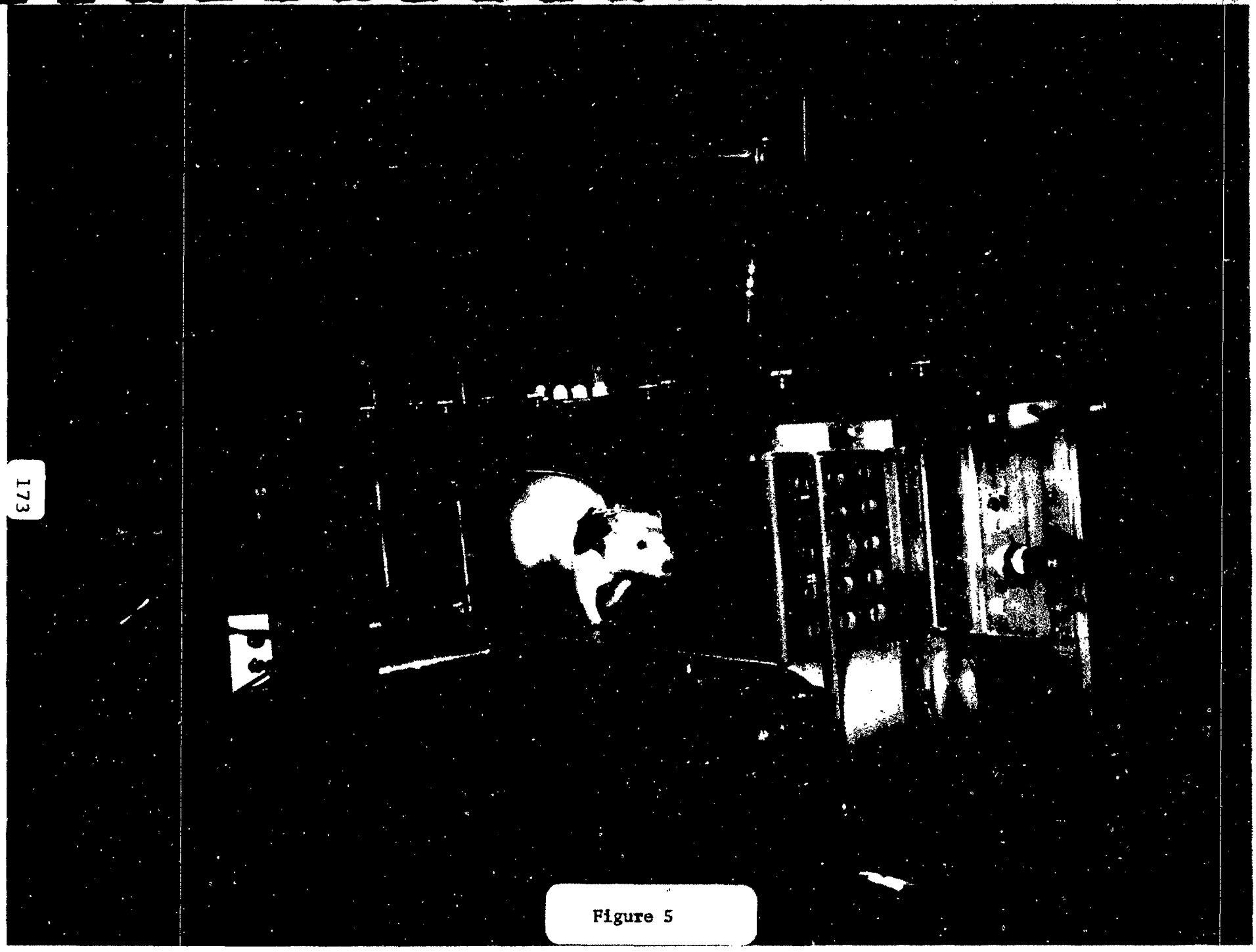


Figure 5

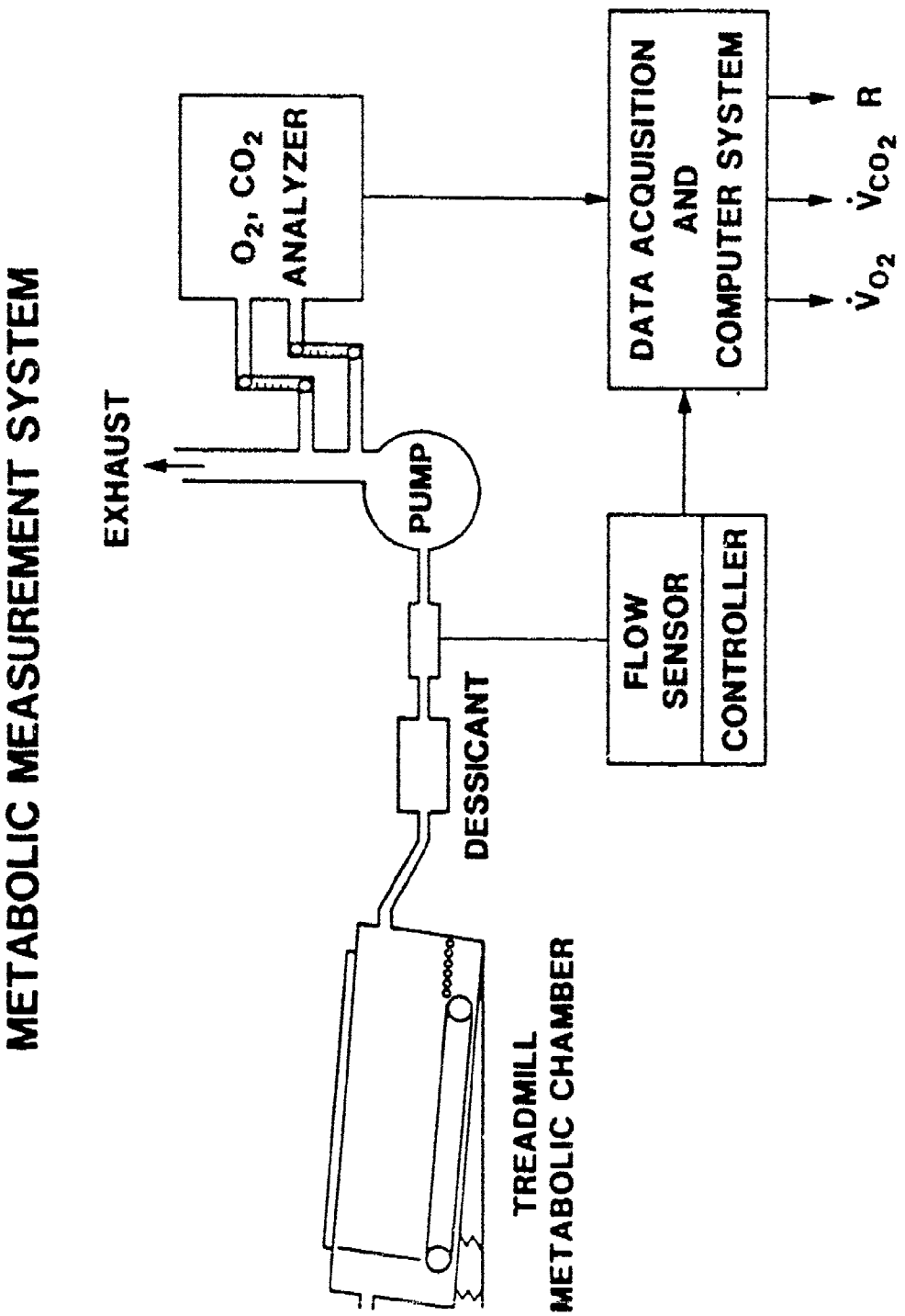

METABOLIC MEASUREMENT SYSTEM
EXHAUST
O_2, CO_2 ANALYZER
PUMP
DESSICANT
TREADMILL METABOLIC CHAMBER
FLOW SENSOR
CONTROLLER
DATA ACQUISITION AND COMPUTER SYSTEM
$\dot{V}_{O_2}$ $\dot{V}_{CO_2}$ R

Figure 6

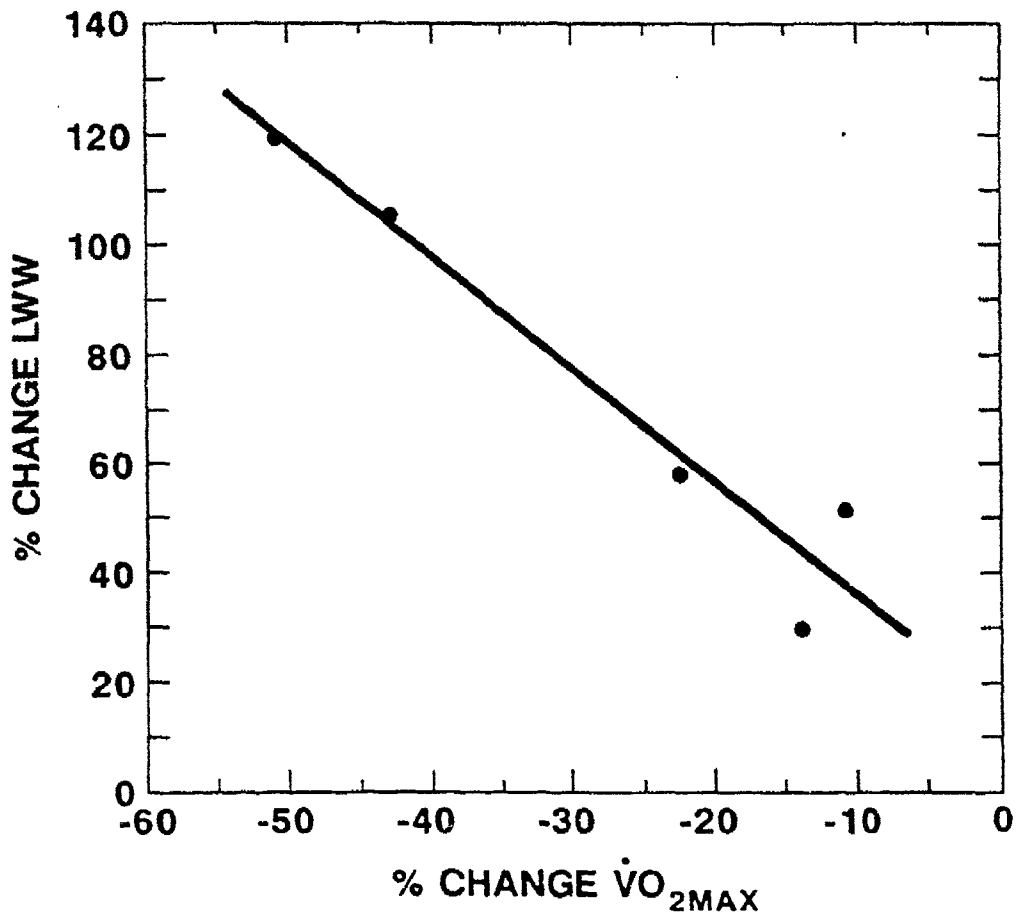

140
120
100
80
60
40
20
0
% CHANGE LWW
-60
-50
-40
-30
-20
-10
0
% CHANGE $\dot{V}O_{2MAX}$

Figure 7

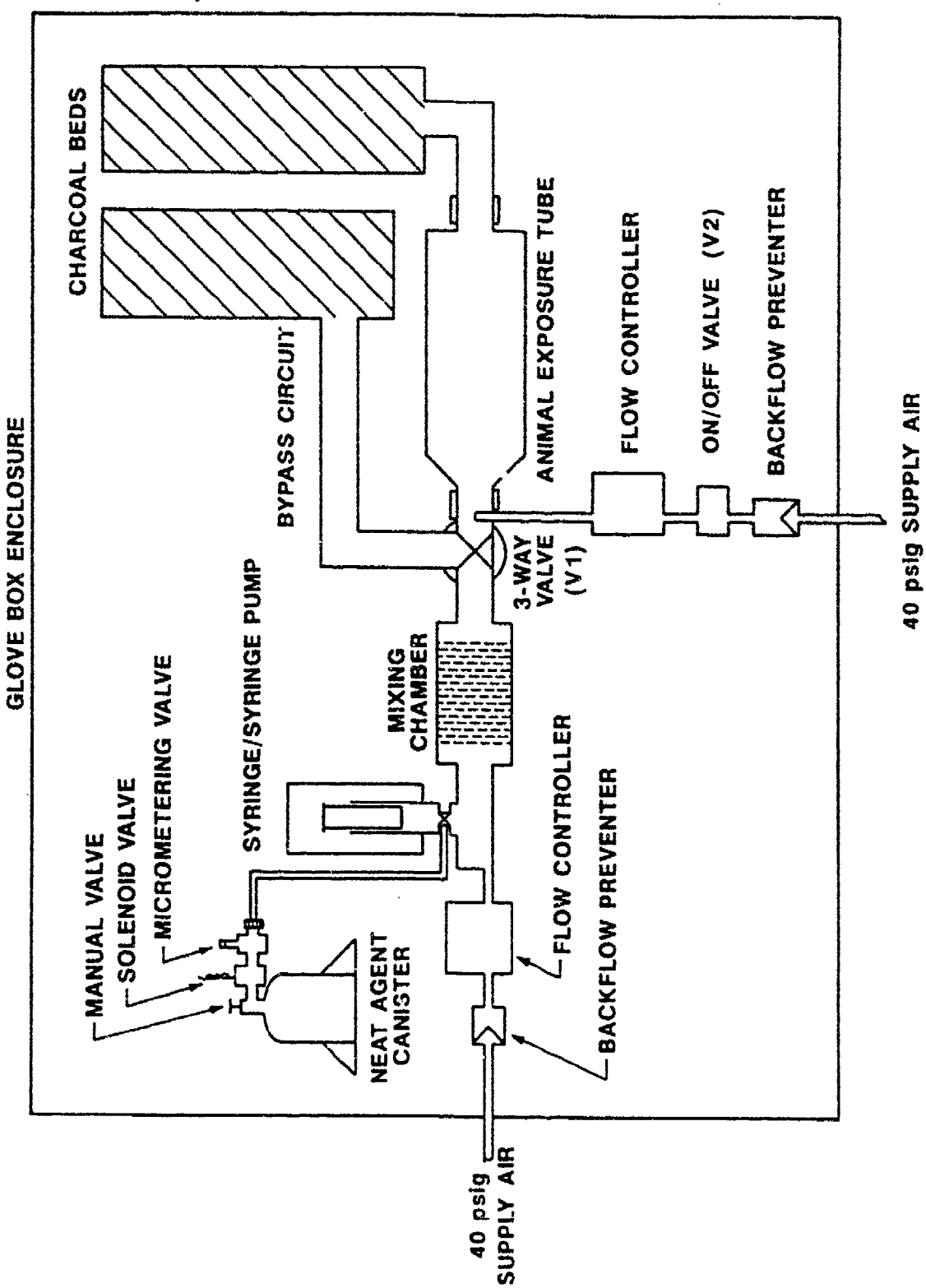

GLOVE BOX ENCLOSURE
CHARCOAL BEDS
BYPASS CIRCUIT
ANIMAL EXPOSURE TUBE
FLOW CONTROLLER
ON/OFF VALVE (V2)
BACKFLOW PREVENTER
40 psig SUPPLY AIR
3-WAY VALVE (V1)
MIXING CHAMBER
SYRINGE/SYRINGE PUMP
MICROMETERING VALVE
SOLENOID VALVE
MANUAL VALVE
NEAT AGENT CANISTER
FLOW CONTROLLER
BACKFLOW PREVENTER
40 psig SUPPLY AIR

Figure 8

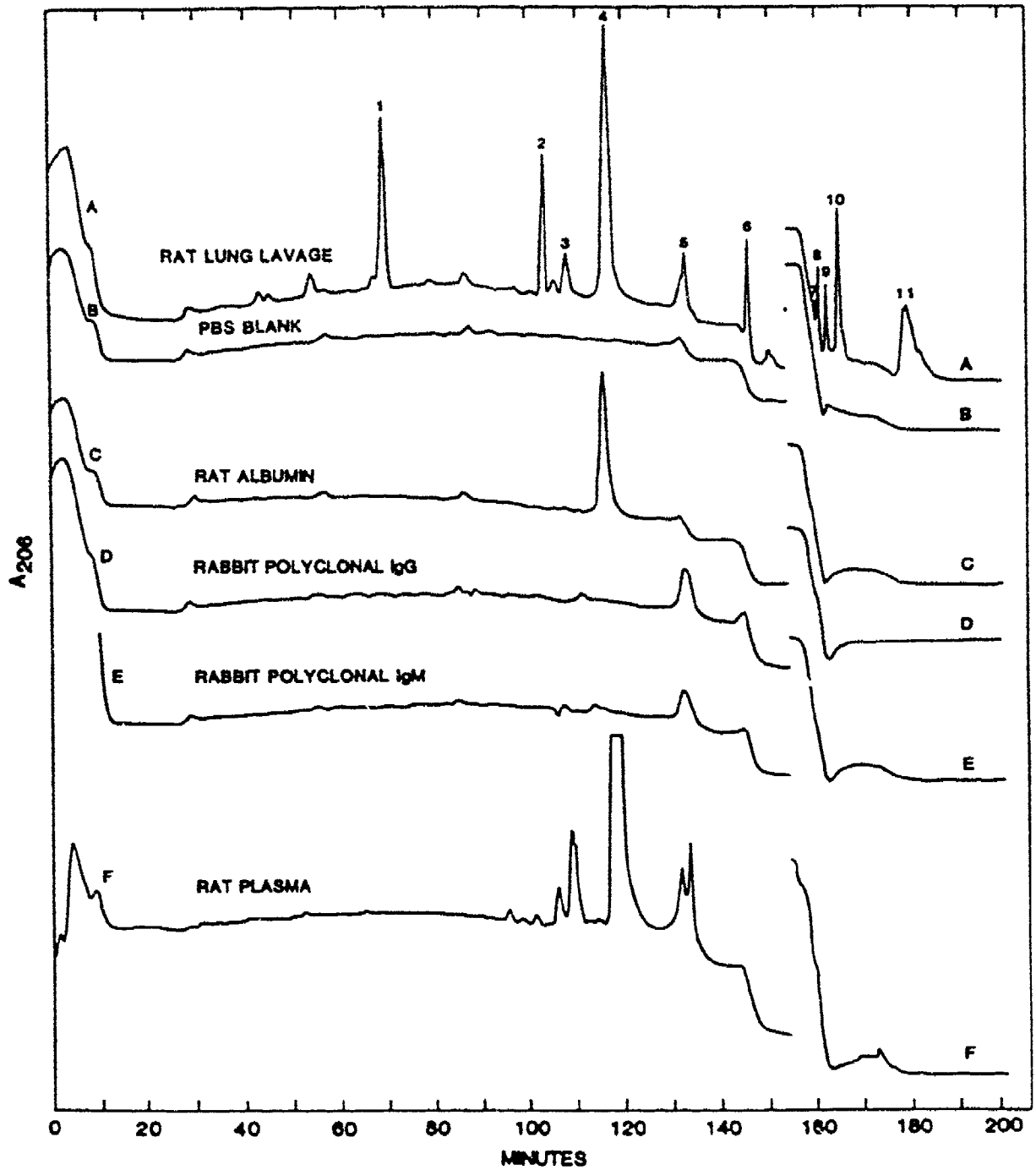

A
RAT LUNG LAVAGE
B
PBS BLANK
C
RAT ALBUMIN
D
RABBIT POLYCLONAL IgG
E
RABBIT POLYCLONAL IgM
F
RAT PLASMA
1
2
3
4
5
6
7
8
9
10
11
A
B
C
D
E
F
A206
0
20
40
60
80
100
120
140
160
180
200
MINUTES

Figure 9

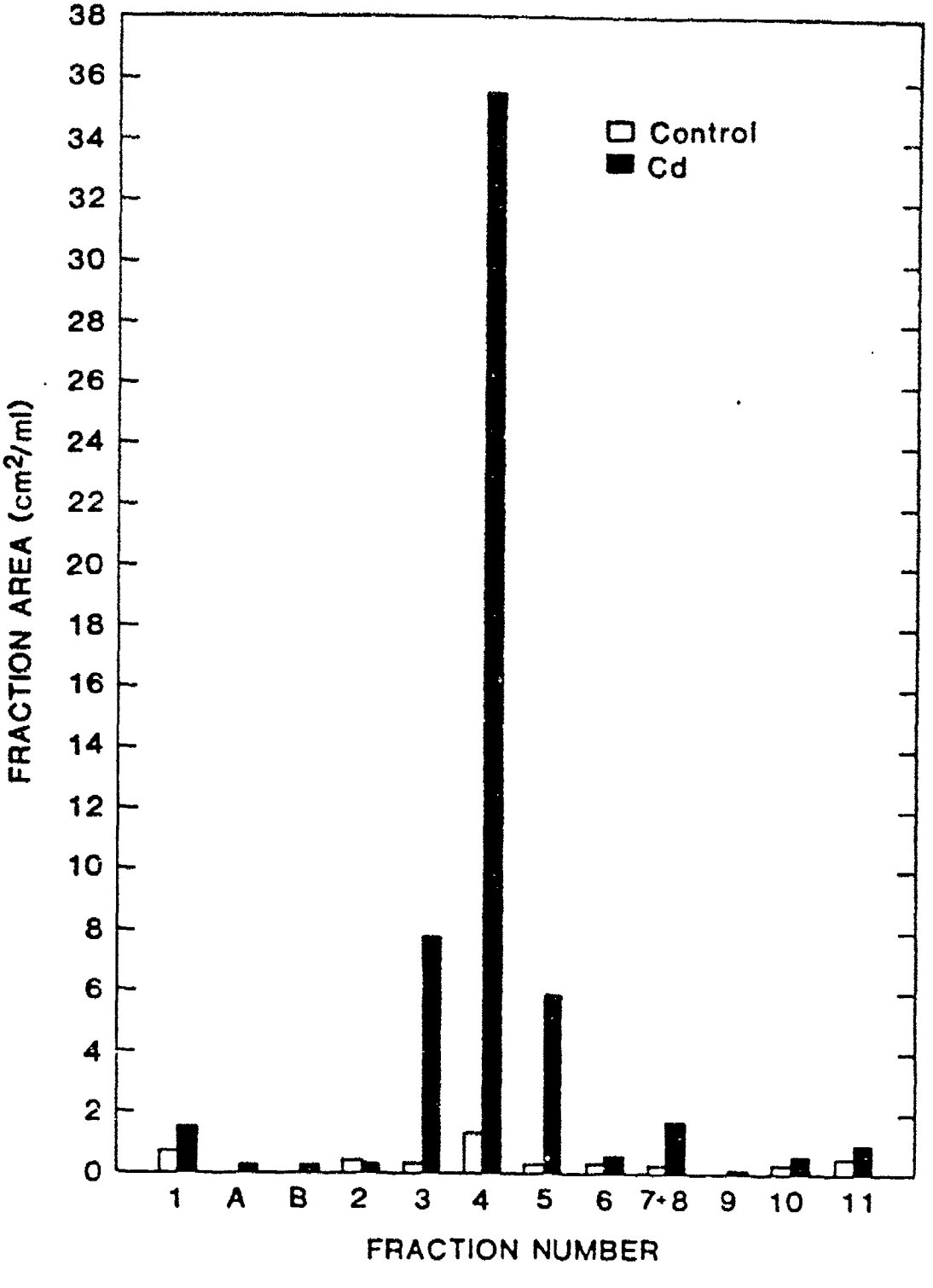

38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
FRACTION AREA (cm²/ml)
Control
Cd
1
A
B
2
3
4
5
6
7+8
9
10
11
FRACTION NUMBER

Figure 10

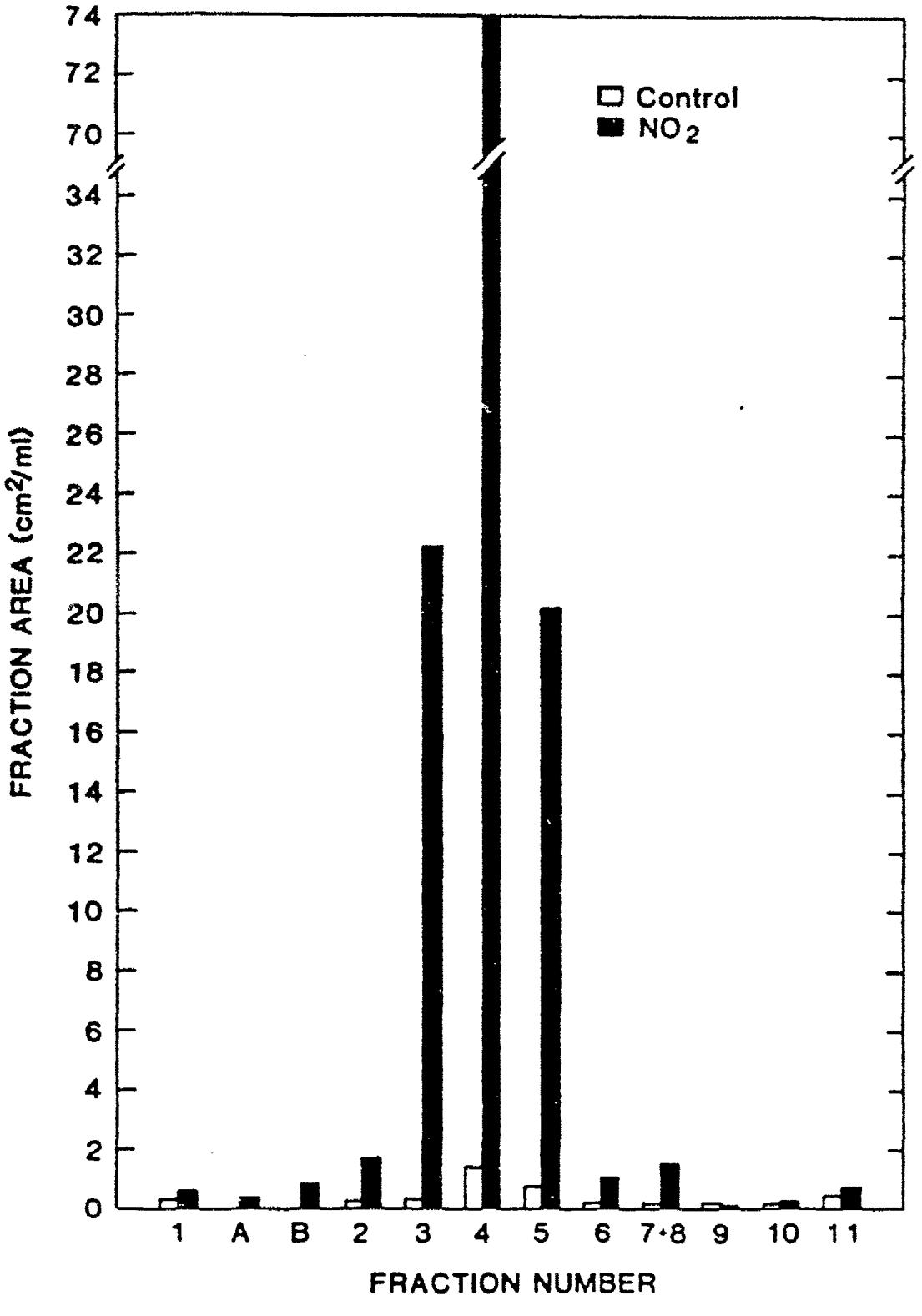

74
72
70
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
Control
NO2
FRACTION AREA (cm2/ml)
1
A
B
2
3
4
5
6
7+8
9
10
11
FRACTION NUMBER

Figure 11

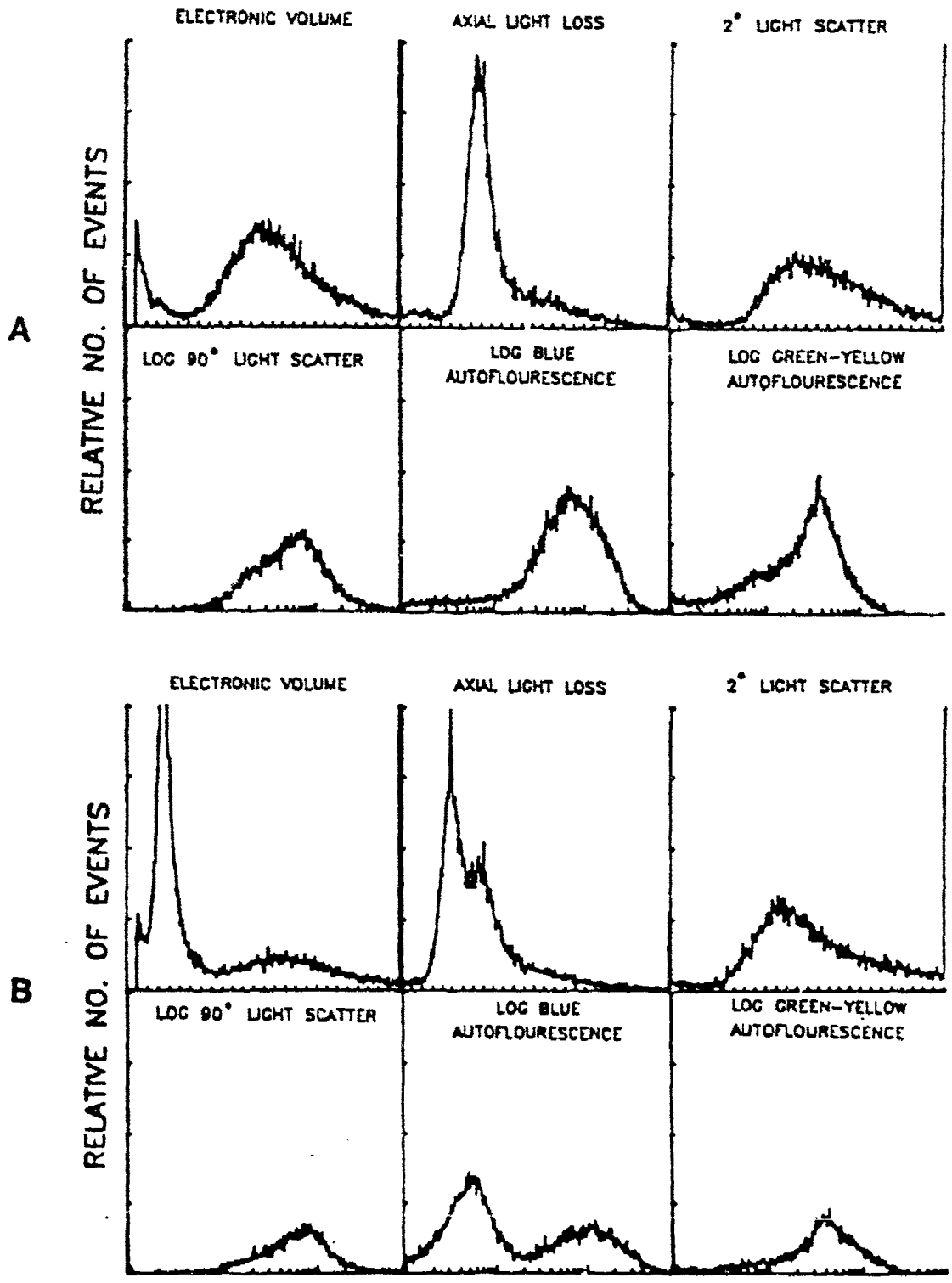


Figure 12A and B

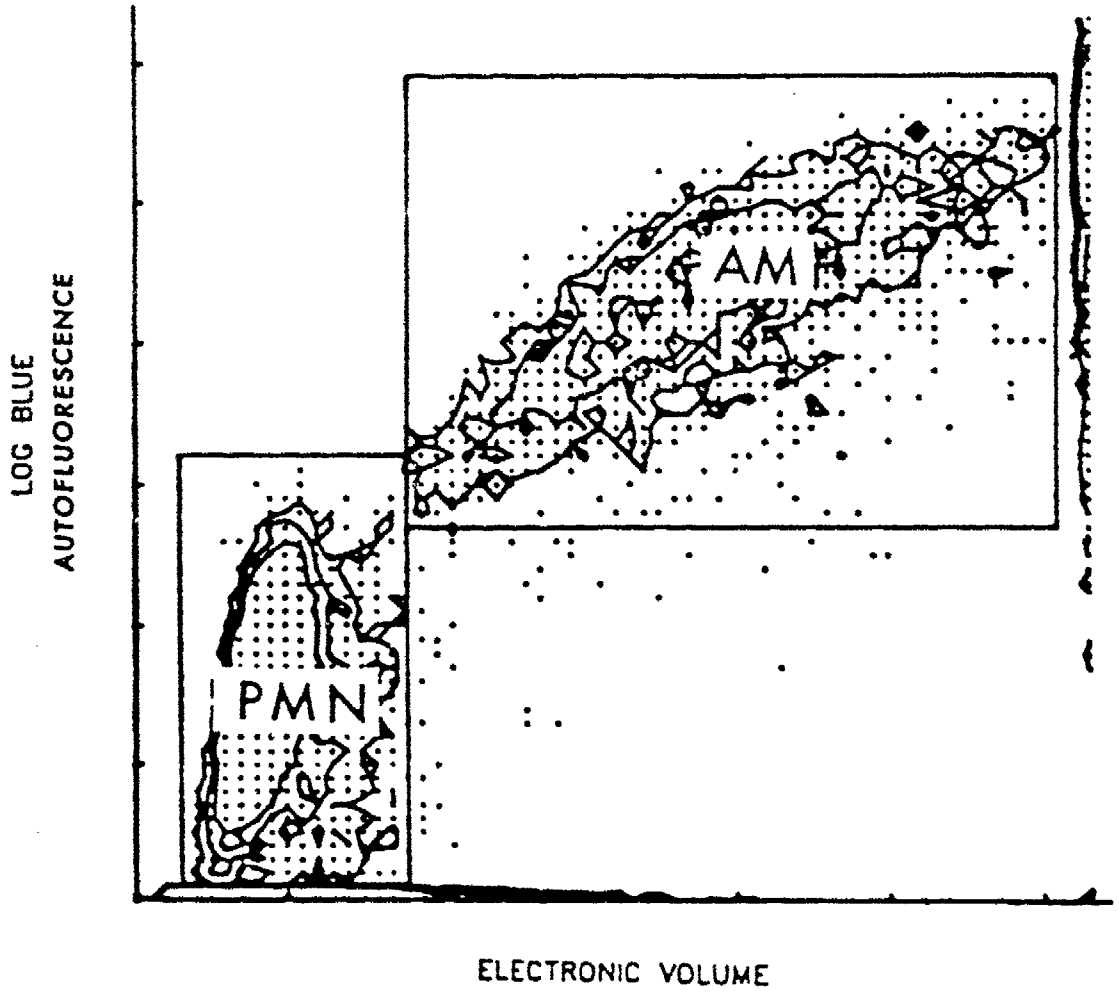


Figure 13

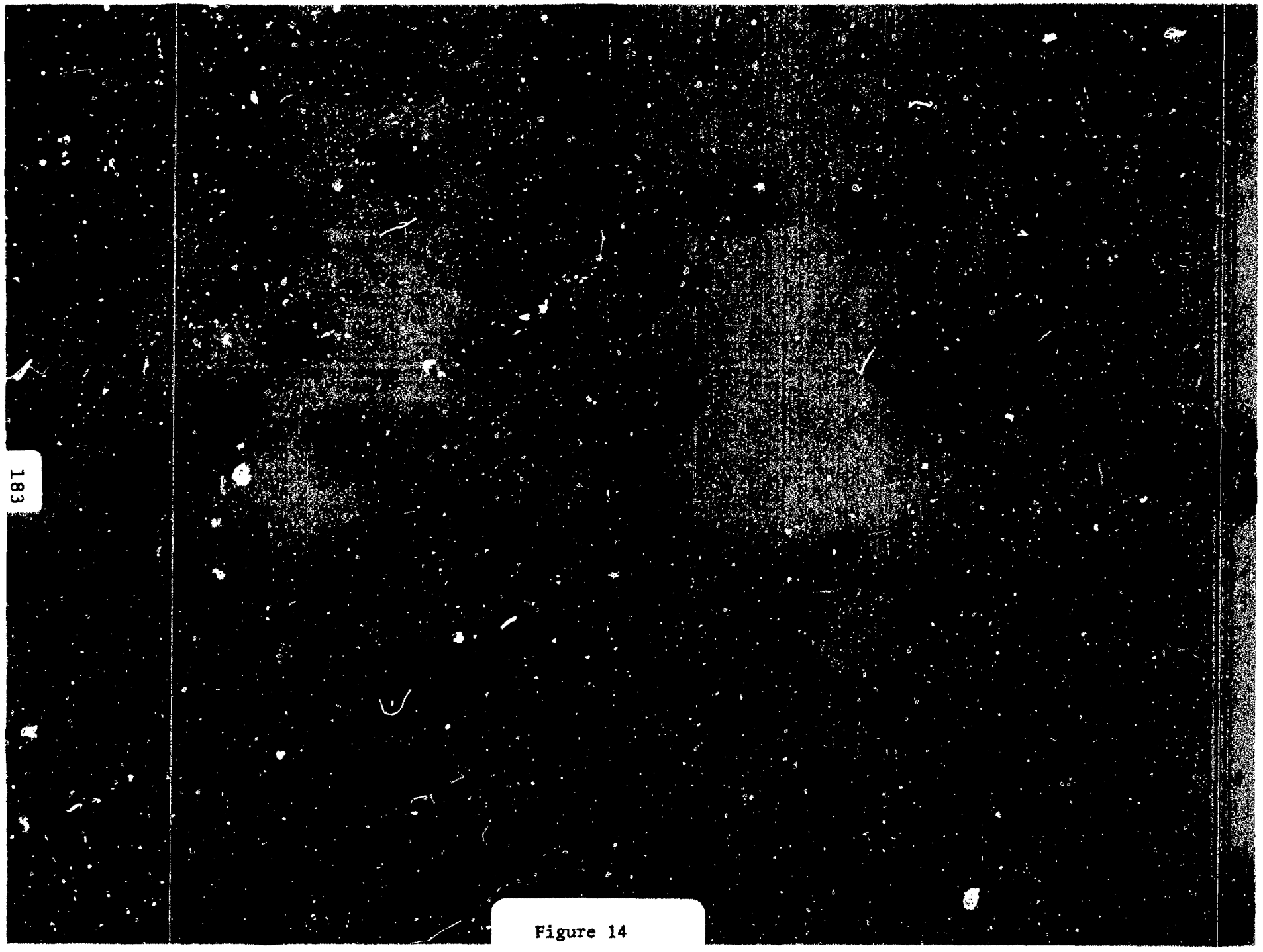


Figure 14

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16. B.E. Lehnert, D.A. Fillak, and C.C. Stewart, Am. Rev. Respir. Dis. 129:A6, 1984.
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19. L.A. Dethloff and B.E. Lehnert, J. Leuk. Biol. 43:80-90, 1988.
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DISCUSSION AFTER DR. LEHNERT'S PRESENTATION

Drs. Mark and Lehnert discussed the versatility of the cell sorter. Dr. Mark asked if it was possible to differentiate between neutrophils in the air space and neutrophils in blood, or between new neutrophils and mature neutrophils. Dr. Lehnert indicated that they had not yet done those experiments but that it was possible to do things such as resolve and sort between peripheral monocytes, interstitial macrophages, and alveolar macrophages. The cells are still alive after being sent through the flow cytometer, which means that it may be possible to reinject them and use them as a biological marker in different lung compartments. Dr. Lehnert then mentioned how the cell sorter can be useful in a situation in which one obtains airway interluminal macrophages from rat tracheal lavage. Also present in lavage samples are exfoliated ciliated epithelial cells, basal cells, and other cells and debris. With the flow cytometer, not only can all the macrophages be differentiated and sorted, but also, almost all of the macrophages in the preparation are recovered and are viable.

Dr. Werrlein asked about the rate of increase of the three factors in the edema fluid after exposure. Dr. Lehnert said that they had not evaluated this and that a radiolabeled albumin was needed to study the kinetics of the albumin change; because at the present time, when looking at albumin and lavage fluid to index permeability pulmonary edema, experimenters don't know if they're looking at "old" albumin or "new" albumin. With the use of radiolabeled blood component constituents, however, it may be possible to see differences in how long-lived these various components are in a particular compartment. Dr. Lehnert added that their current process of isolating pure fractions of alveolar macrophages and PMNs is very labor-intensive, requiring several experiments to get the methodology established. The rate-limiting step is the actual sorting of cells--with their present methodology, they can acquire approximately 2 million cells per day. As such, the material is precious and requires the development of microassays.

Dr. Werrlein asked if the experimental animals are generating the same vascular pressure or if the resultant edema is a combination of pressure and permeability. Dr. Lehnert responded that this is the heart of the issue, and the problem is that scientists don't know all of the physiological and physical processes involved in the development of this type of edema, let alone the cellular and molecular mechanisms involved in this process. As an example, he discussed problems in isolating the specific site of the membrane leak. (That is, the leak resulting in the accumulation of fluid in the airways.) There is no proof

that the fluid originates in the space in which it is found, and retrograde movement of fluid may be involved. There are a variety of physiologic processes involved, but as yet it is not known which compartment contains the leak. In addition, the translocation of constituents from the blood compartment may serve as a signal resulting in a secondary series of events which actually account for the injury itself.

Dr. Fowler noted that it was quite a novel finding that exercise amplifies the permeability edema--expecially considering that the concept of permeability pulmonary edema itself was not even established in 1970. Dr. Lehnert responded that there is a need to know more about the status of the cell populations in the lungs, stating that in his HPLC profile they're able to identify eight proteins unique to the lung compartments. Currently however, it requires too much time (260 minutes) to easily run such a profile by HPLC, and better methods are needed. Dr. Mark inquired if high-altitude pulmonary edema was a good clinical example of permeability edema. Dr. Fowler indicated that a number of investigators (such as the group led by Dr. West), have found that they could potentiate the hypoxia and clinical signs and effects of high-altitude edema with exercise. However, it has not been established that this is a true permeability-type pulmonary edema. COL Dunn noted that as early as World War I it was known that exercise after exposure to phosgene exacerbated the lung damage, but that the additional injury was not recognized as permeability edema. Dr. Lehnert thought that scientists should have guessed that this phenomenon would occur based on the facts that by simply increasing the expired respiratory minute volume in sheep, experimenters could provoke changes in the fluid compartments of the lung, and that in distance runners there is a proportionality (during exercise) between diffusion capacity and cardiac output. At rest this proportionality relationship is uncoupled, and has been interpreted as representing a subclinical low-grade pulmonary edema.

The discussion continued with Dr. Lehnert commenting on the fact that the subsidence kinetics of pulmonary edema following immediate post-[NO_2]exposure exercise versus immediate plus 8-hour post-exposure exercise differ. Results [from Dr. Lehnert's laboratory] seem to point to the translocation of water and blood solutes into at least two compartments that have differing rates of resolution of edema fluid. Exercise immediately after NO_2 exposure appears to result in the further filling of one of these compartments with edema fluid beyond the level produced by NO_2 exposure followed by rest. This potential compartment evidently continues to fill for at least 24 hours post-exposure. On the other hand, a second, more rapidly resolving compartment accumulates fluid during or shortly after a second exercise bout. Dr. Lehnert speculated that the immediate

post-NO_2 exposure exercise period resulted primarily in the further filling of the lung's interstitium with fluid, whereas the second exercise period mainly promoted the translocation of edema fluid from the already well-filled interstitium into the more rapidly clearing alveoli. This phenomenon remains to be fully explained and probably involves the biophysics of fluid mechanics.

These comments were followed by a brief discussion by Drs. McMaster, Werrlein, Stavert, and Lehnert, and MAJ Ripple in which they debated the merits of examining the effects of training exercise, noting that after long periods of conditioning, enzyme levels (e.g., LDH levels) may change in runners, and that VO_{2max} doesn't stabilize for many months in runners under some conditions. In other words, there may be an adaptation effect to the exercise regimen in animals which might influence the variables being measured.

PULMONARY EDEMA IN A CHRONICALLY INSTRUMENTED SHEEP MODEL

Dr. Frederick L. Glauser

The purpose of this presentation was to describe the methods to be used to maintain a chronically instrumented sheep model for examining the long-term effects of inhalants. Once established, the model would be used for the examination of sheep exposed to the organohalides PFIB and TFD.

The model was similar to the one described by LTC Jaeger. After surgical preparation and recovery, the awake, unanesthetized sheep could be observed for responses that should be similar to those expected in humans. Data on baseline hemodynamics and respiratory mechanics could be obtained, lymph flow and fluid flux could be measured, and lymph fluid cells could be separated by cytospin methods.

An example of the application of this system was given. Interleukin II is used clinically for some types of cancer. However, its usage is frequently associated with side effects, including pulmonary edema. The objective of this study was to determine if the pulmonary edema was cardiogenic or noncardiogenic. Using the sheep model described above, it was found that lymph flow increased after interleukin-II, but that the lymph/plasma protein ratio did not change, suggesting the occurrence of noncardiogenic pulmonary edema.

PULMONARY EDEMA IN A CHRONICALLY INSTRUMENTED SHEEP MODEL

Dr. Frederick L. Glauser and
B. Smith-E-Incas

CHRONIC SHEEP LUNG LYMPH FISTULA MODEL

ADVANTAGES

Awake, Unanesthetized animals can be used
Lab has Extensive Experience
'Pure' Lung Lymph
Responses Similar to Humans

DISADVANTAGES

'Impure' Lung Lymph
Responses Different from Humans
Intravascular Macs [Macrophages]

MODEL PREPARATION

1. Anesthetize, intubate, ventilate
2. Percutaneous insertion of carotid artery, PA catheters
3. Recovery
4. Next day - thoracotomy
   Caudal mediastinal lymph node catheter
5. 2-4 day recovery in special cage

INCLUSION CRITERIA

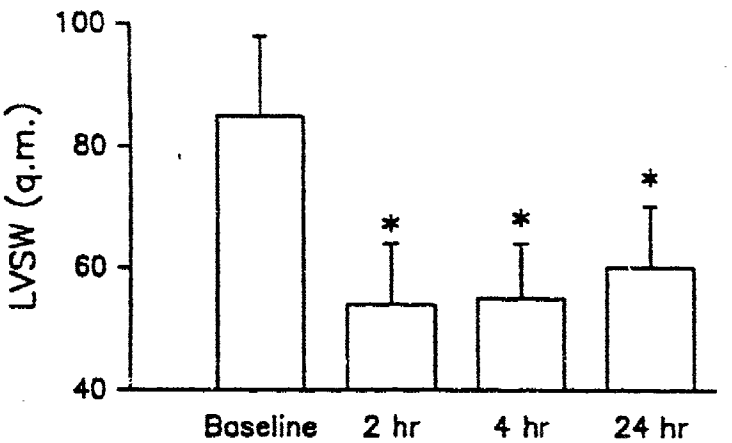
1. SBP > 80 mm Hg
2. PaO_2 > 90 TORR
3. PAP < 20 mm Hg

MEASUREMENTS

HEMODYNAMICS

Direct - SBP, PAP, PWP
RAP, CO, HR

Calculate - SVR, RVSW, LVSW



RESPIRATORY/GAS EXCHANGE

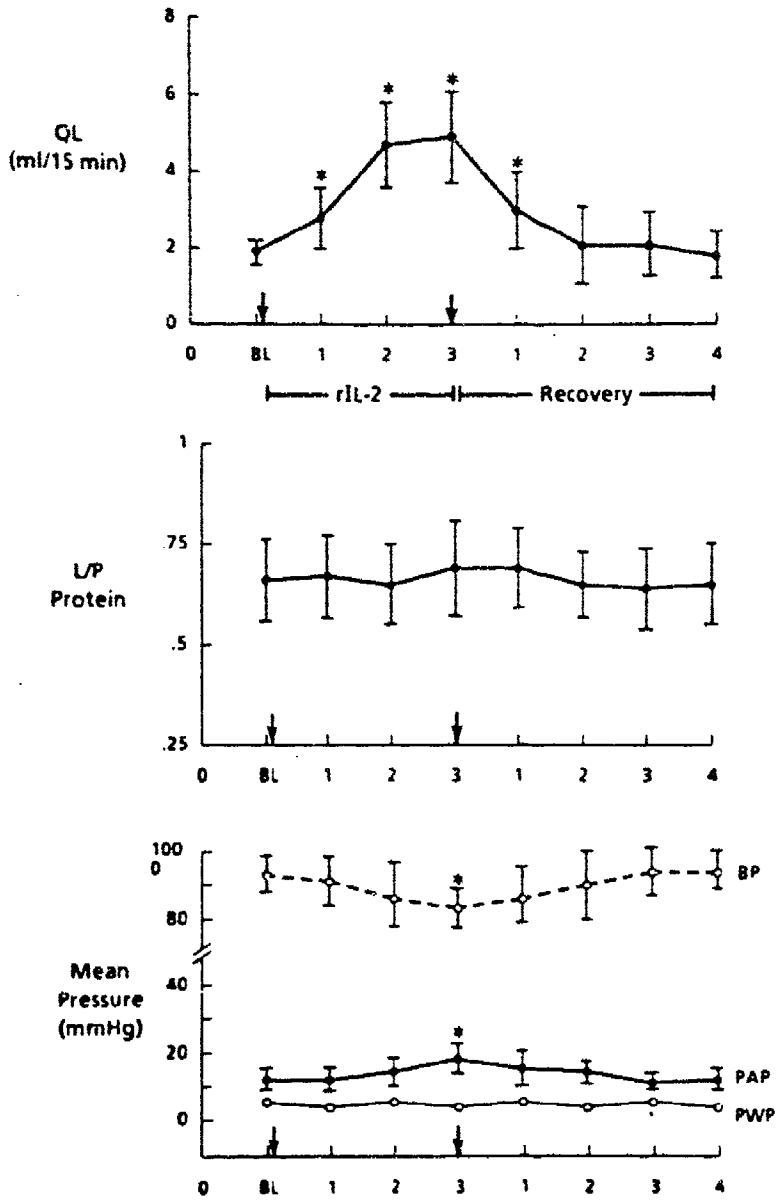
Direct - ABGs, Mixed Venous Blood Gases, RR

Calculate - A - aO_2, a/AO_2, (VD/VT)

CAUDAL MEDIASTINAL LYMPH

Direct - QL, Total Proteins, Cytospin

Indirect - L/P Total Protein, Protein Clearance



PROTOCOL USING THE CHRONIC SHEEP LUNG LYMPH FISTULA MODEL

1. Baseline:
   - Hemodynamics
   - Respiratory
   - Blood
   - Lymph
   - BAL
2. Exposure
3. Repeat measurements:
   - Hours, days
4. Monitor recovery

TIME COURSE/EXTENT OF INHALANT LUNG INJURY

Glauser:
- Hemodynamics
- Respiratory
- Gas Exchange
- QL, L/P Protein

Fowler and Bechard:
- Lymph, Blood

Kramer and Fowler:
- BAL AM

ONGOING QUESTIONS: INHALANT LUNG INJURY

1. Specific inhalant; dose; exposure duration
2. Apparatus for inhalant administration
3. Appropriate time points for data acquisition

DISCUSSION AFTER DR. GLAUSER'S PRESENTATION

Dr. Lehnert noted that relatively little was known about the endothelial cells lining the lymphatic channels, and asked where the lymphatic channels actually began in the peripheral region of the sheep lung. Dr. Glauser said that this was in the thick part of the interstitium, at the terminal bronchiole level. He added that because there is a pressure gradient (i.e., there is negative pressure in the lymphatic channels themselves), various blood elements can move through the loose interstitium into the lymphatics. This was significant because, if the lymphatics were blocked, you could conceivably have a variety of cell changes and pulmonary edema which would not be easy to recognize.
Dr. Glauser also noted that one problem with the model was that the lymph nodes themselves may be able to alter the "cell traffic," i.e., the influx and outflux of the lymphocytes. This was something, Dr. Glauser suggested, that needed to be evaluated further.

Dr. Mark mentioned that in humans there are cell-staining differences between lymph and capillary endothelial cells, and asked if there were other differences between the two cell types. Dr. Lehnert responded that the older literature alluded to the contractile nature of the lymphatic channels. Dr. Glauser said that he thought that lymphatic channels probably were contractile, and that one indication of this was the observation of pulsatile flow through the lymphatics which could be influenced by the respiratory rate itself.

INHALED VESICANTS: ASSESSMENT OF DIRECT PARENCHYMAL LUNG INJURY USING BRONCHOALVEOLAR LAVAGE

Dr. Alpha A. Fowler

These proposed studies will use repetitive bronchoalveolar lavage (BAL) to assess the extent of lung injury following exposure to inhaled pulmonary toxicants. The methods will allow (a) documentation of changes in alveolar cell profiles; (b) characterization of the severity of the injury to the alveolar-capillary membrane by BAL analysis of the total protein content and by tracking the movement of progressively larger proteins into the alveoli (using molecular sieving techniques); (c) documentation of the extent of functional alterations of alveolar macrophages; (d) characterization of the extent to which arachidonate metabolites (which are found in the air space as a result of the exposure challenge) participate in the injury mechanism; and (e) documentation of the potential efficacy of various agents (such as arachidonate metabolite inhibitors and histamine receptor antagonists) in attenuating the lung injury.

The instrumented sheep model (as described by Dr. Glauser) will be used in these studies. Several notable features of the methodology are the use of a specially designed fiber-optic bronchoscope to perform BAL in animals with long tracheas, and the fact that each animal will serve as its own control--one lung will be used to obtain baseline data and the contralateral lung will be used to determine effects of exposure to the agent of interest.

A previous ovine study demonstrated the validity of this model system. Anesthetized, intubated sheep were exposed to ethchlorvynol (ECV) and then submitted to repetitive BAL. Results included the observation that BAL protein was significantly elevated 60 minutes after exposure to ECV. Another important finding was the striking increase in neutrophils 60 minutes after exposure (previous presentations had implicated these cells in the mechanism leading to ECV-induced lung injury).

INHALED VESICANTS: ASSESSMENT OF DIRECT PARENCHYMAL LUNG INJURY USING BRONCHOALVEOLAR LAVAGE

Dr. Alpha A. Fowler and B.J. Fisher

ROLE OF ARACHIDONIC ACID METABOLITES

STUDY GOALS

- Employ bronchoalveolar lavage (BAL) to document extent of lung injury following airway exposure to inhaled agents.
- Document changes in alveolar cellular profile following airway challenge.
- Characterize severity of injury to the alveolar-capillary membrance (gas exchange surfaces) by analyzing BAL total protein content and performance of molecular sieving studies (polyacrylamide gel electrophoresis), radial immunodiffusion studies.
- Document extent of functional alteration of alveolar macrophages due to exposure.
- Characterize the extent to which proinflammatory lipids (arachidonate metabolites) participate in this form of lung injury.
- Document potential efficacy of specific agents (i.e., arachidonate metabolite inhibitors, pentoxiphylline, H_1, H_2 receptor antagonists) in attenuating lung injury.

PROTOCOL

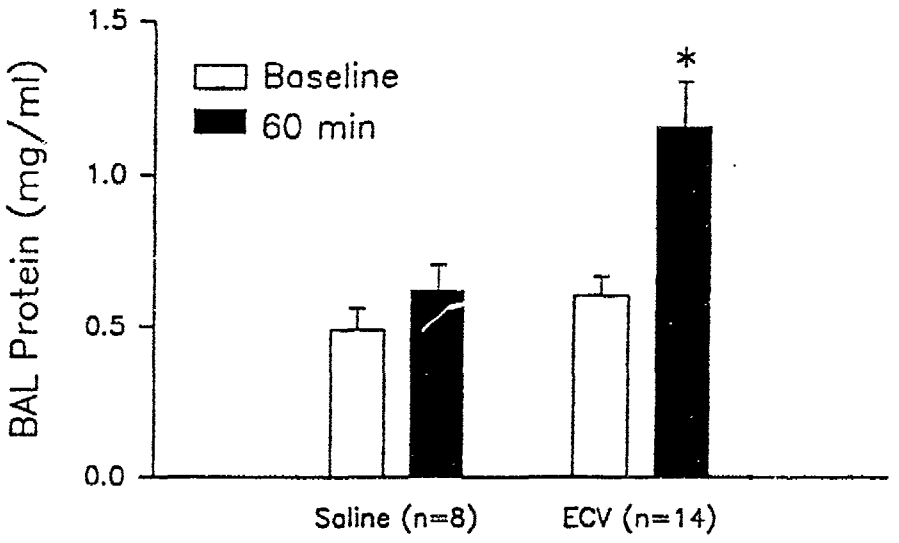
- Inhalation exposure of study animal via approved apparatus.
- Following exposure physiologic profiles will be followed to determine deteriorating gas exchange status.
- Bronchoalveolar lavage performed to document intensity of inflammatory response at select time points following exposure.
- Acute post-exposure and "chronic" post-exposure studies planned to establish acute and prolonged effects.

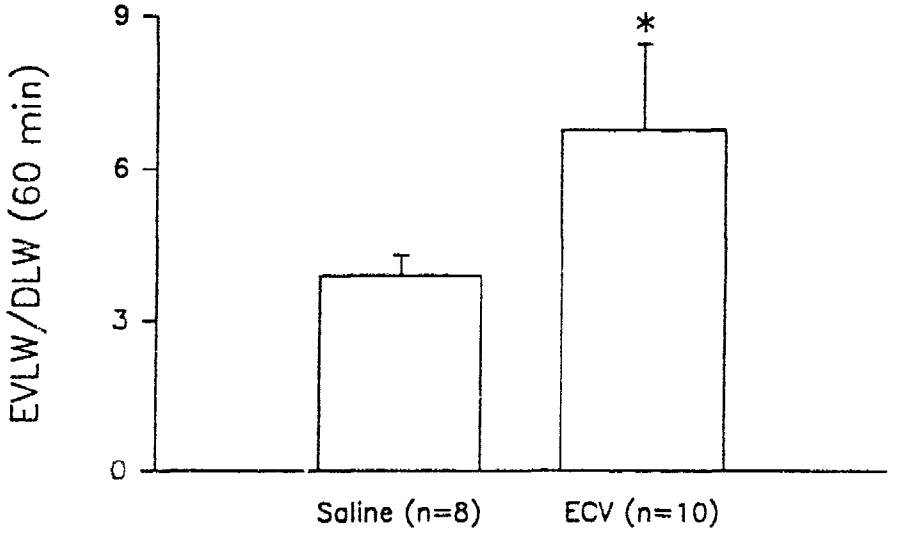
OVINE BRONCHOALVEOLAR LAVAGE

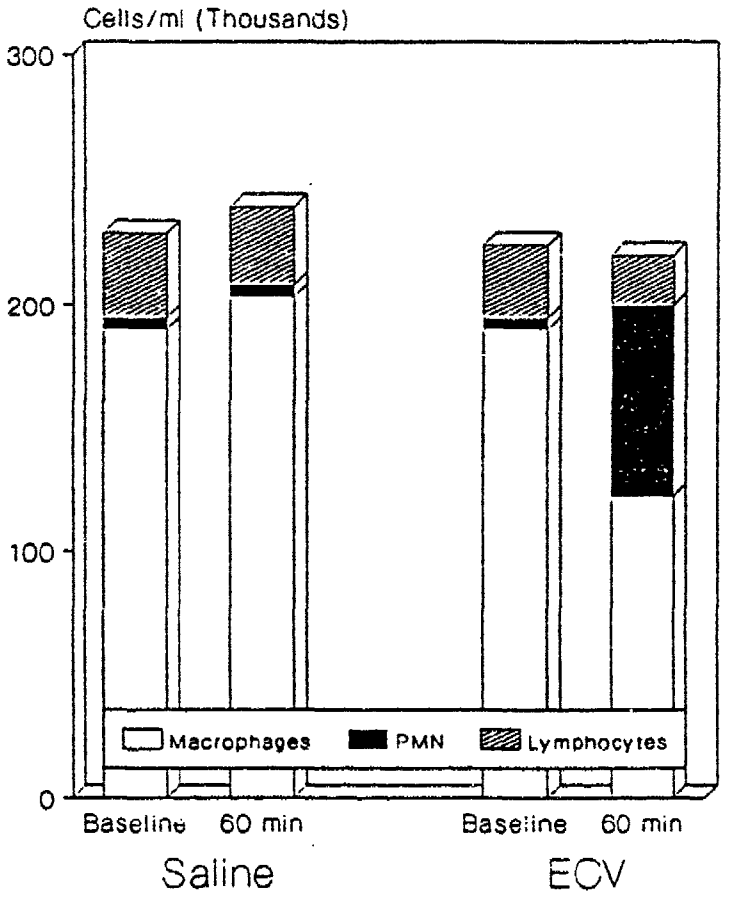
- Technique/Instrumentation
- Application of BAL in Sheep (previous studies)

PRIOR OVINE STUDIES: LUNG INJURY DUE TO ETHCHLORVYNOL INFUSION

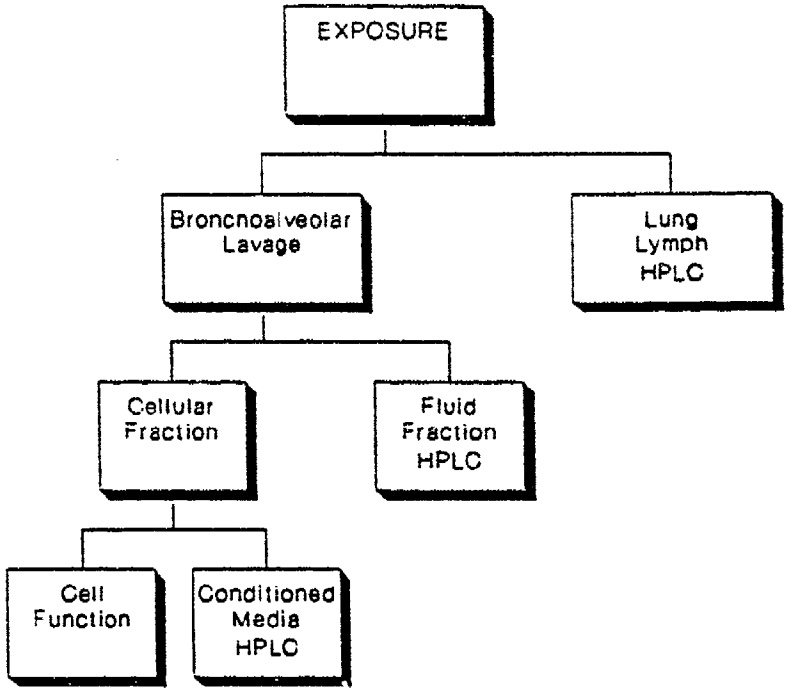
- Sheep Anesthetized, Airway Intubated
- Invasive Monitoring Accomplished
- BAL Performed at Baseline
- ECV Infused
- BAL Repeated at 1 hour


1.5
1.0
0.5
0.0
BAL Protein (mg/ml)
Baseline
60 min
*
Saline (n=8)
ECV (n=14)


9
6
3
0
EVLW/DLW (60 min)
*
Saline (n=8)
ECV (n=10)


Cells/ml (Thousands)
300
200
100
0
Macrophages
PMN
Lymphocytes
Baseline
60 min
Baseline
60 min
Saline
ECV

Arachidonate Metabolites



ENDOTHELIAL CELL STUDIES: DOES AIR SPACE EXPOSURE PROMOTE ENDOTHELIAL CELL INJURY?

Dr. Alpha A. Fowler

These studies would focus on the phenomena in the air space which serve to promote endothelial cell injury, and on whether intervascular effector cells have a role in this process. These efforts would (a) determine whether airway exposure to toxicants alters phagocyte interactions with the vascular endothelium; (b) determine if there are direct effects of toxicants on endothelium; (c) observe the effects of post-exposure biological fluid (BALF, lymph, plasma) products on endothelial cell function; and (d) determine if *in vitro* toxicant-exposed effector cells (such as PMNs) promote endothelial cell injury.

One phase of this project would involve *in vitro* endothelial cell permeability assays, in which toxicants or isolated fluid constituents would be tested for their ability to induce alterations in permeability across endothelial cell monolayers. A commercially available cell culture system with a polycarbonate membrane fixed to the bottom of the well chamber would permit the study of transport dynamics on both sides of a cell monolayer grown on the membrane. Data were shown depicting the effects of recombinant Interleukin-II on the transport of 125-I-labeled albumin across an endothelial cell monolayer (Interleukin-II did not promote transport of albumin).

Finally, plans were discussed to study a model which would involve adherence of neutrophils to the endothelial cell monolayer, a system which could provide information about the effects of the neutrophil on the injury process.

ENDOTHELIAL CELL STUDIES: DOES AIR SPACE EXPOSURE PROMOTE ENDOTHELIAL CELL INJURY?

Dr. Daniel E. Bechard and R.B. Coles

(Presented by Dr. Alpha A. Fowler)

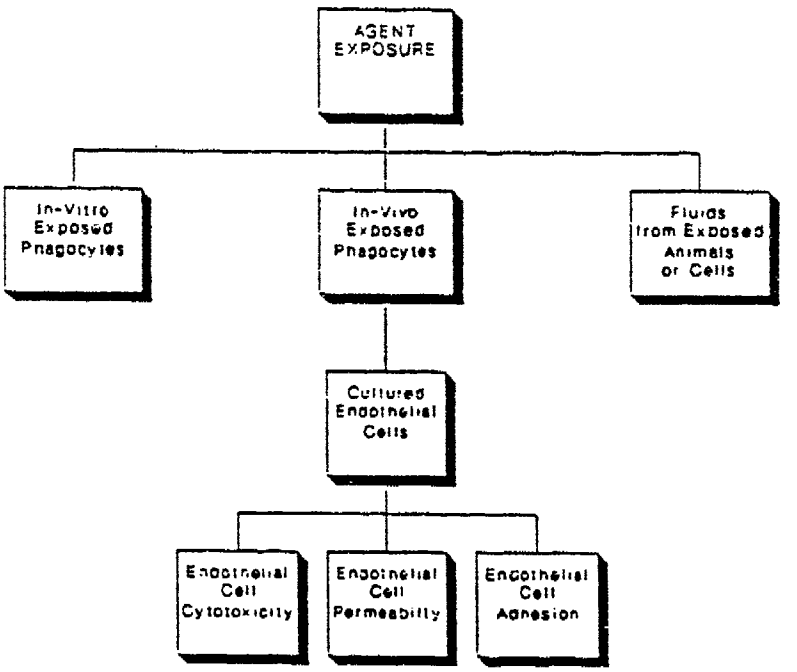
STUDY GOALS

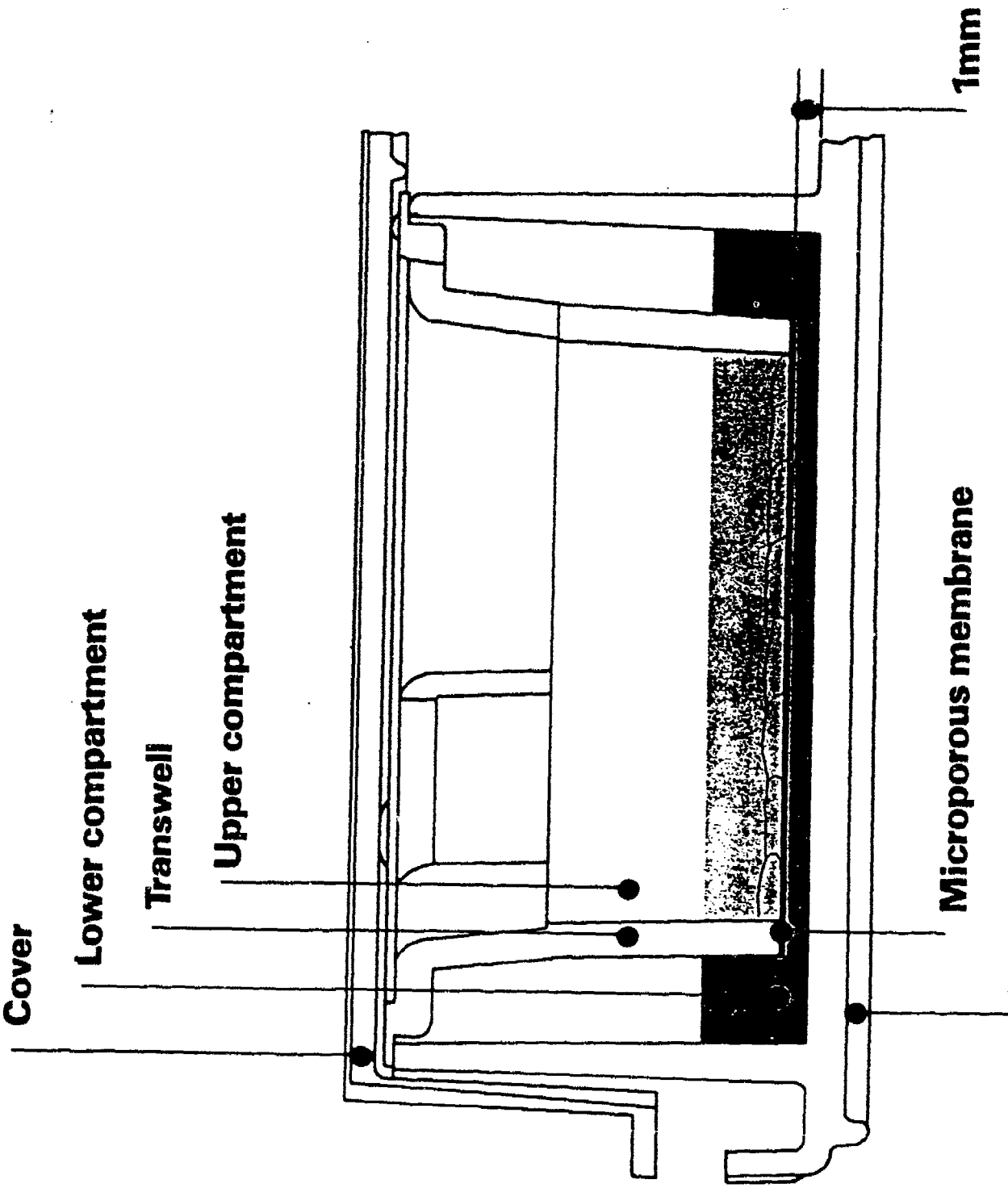
- Determine whether airway exposure to agent alters phagocyte interaction with vascular endothelium.
- Determine direct effects of agent upon endothelium.
- Determine effects of post-exposure (non-cellular) biological fluids (i.e., BALF, lymph, plasma) upon endothelial cell function.
- Determine whether *in vitro* agent-exposed effector cells (PMN, lymphocytes, monocytes) produce endothelial cell injury.

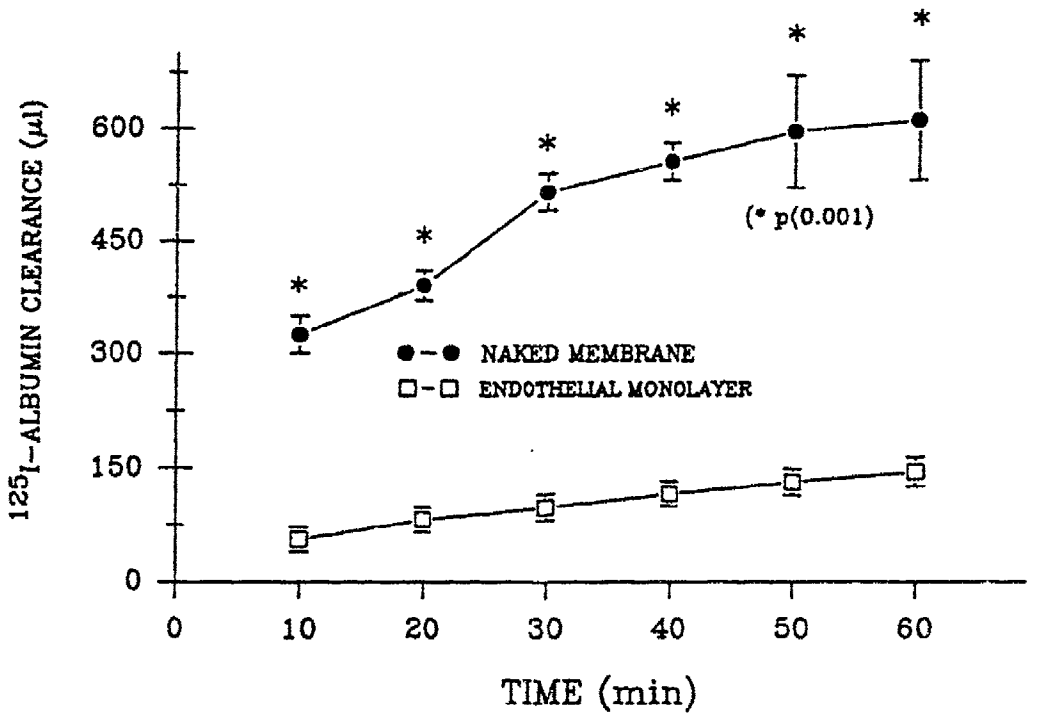
STUDIES PLANNED

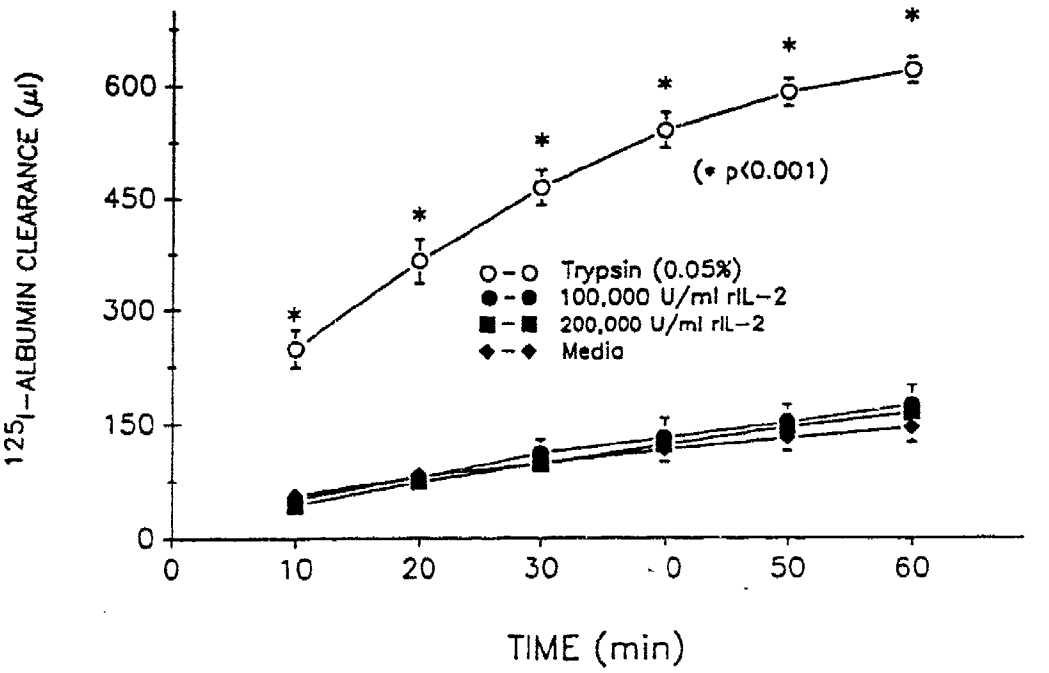
- *In vitro* cytotoxicity studies: Effector cells (PMN, lymphocytes, monocytes) obtained from agent-exposed animals will be studied for their ability to produce nonspecific cytotoxicity (^{51}Cr cytotoxicity assay).
- *In vitro* endothelial cell permeability assay: Agent (and biological fluids from exposed animals) will be tested for their ability to induce alterations in permeability across endothelial cell monolayers.
- *In vitro* endothelial cell adhesion studies: Assess adhesion of PMN, monocytes, and lymphocytes to endothelial cells following exposure to agent.

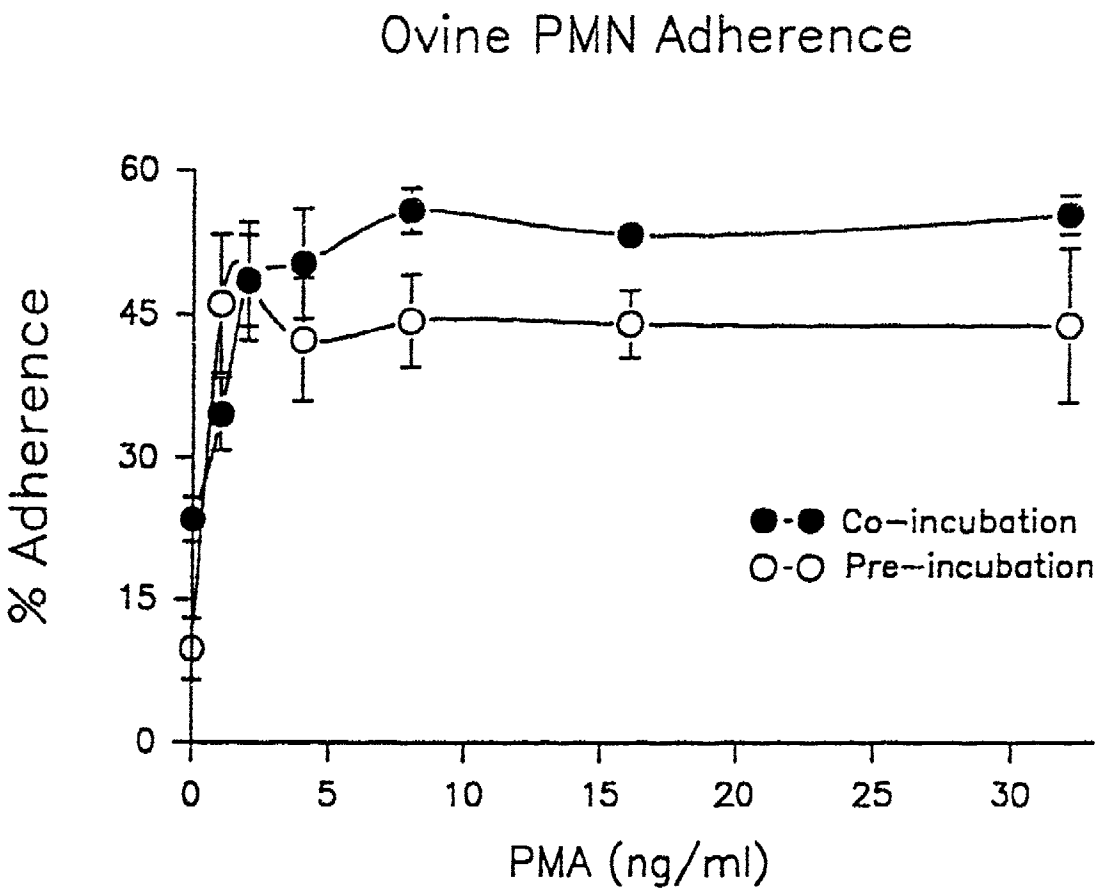
Endothelial Cell Studies




Cover
Lower compartment
Transwell
Upper compartment
1mm
Microporous membrane
6 well cluster plate


^{125}I-ALBUMIN CLEARANCE (µl)
600
450
300
150
0
0
10
20
30
40
50
60
TIME (min)
(* p(0.001)
NAKED MEMBRANE
ENDOTHELIAL MONOLAYER


^{125}I-ALBUMIN CLEARANCE (µl)
600
450
300
150
0
0
10
20
30
0
50
60
TIME (min)
(* p<0.001)
Trypsin (0.05%)
100,000 U/ml rIL-2
200,000 U/ml rIL-2
Media


Ovine PMN Adherence
% Adherence
60
45
30
15
0
0
5
10
15
20
25
30
PMA (ng/ml)
Co-incubation
Pre-incubation

DISCUSSION AFTER DR. FOWLER'S PRESENTATIONS

Dr. Lehnert began the discussion by noting that in a leaky lung it's difficult to exclusively sample the conducting airways in the alveolar epithelial surface. That is (if the lung is leaky after injury), it is still possible that samples collected from the alveolar space are contaminated with constituents from the plasma and interstitial compartments. Dr. Fowler noted that this was a good point--and that is why all three compartments must be sampled or monitored in some fashion. He went on to say that after injury to the alveolar capillary membrane there is entry of plasma proteins into the alveolar capillary space, and that in his laboratory they have the capacity to use the lung-lymph fistula model. Further, the sheep model is particularly useful because with it, you can sample the interstitium. Dr. Lehnert stated that the use of lavage may itself promote the contamination of the sample by drawing in extra-alveolar constituents. Dr. Mark then entered the discussion, noting that, in human lung disease, neutrophils are always present in the lavage, and that clinicians rely on that presence as a marker of injury; pathologists, however, never see neutrophils. He added that, as it turns out, the neutrophils come from the bronchiolar level and not the alveolar level, which is significant in that it indicates that alveolar lavage definitely contains contaminating constituents, particularly when it is done in leaky lungs.

Dr. Lehnert then raised the issue of involvement of intravascular macrophages in the inflammatory process, and of whether this was pertinent in the sheep model. Dr. Fowler noted that although these are attached cells (whose numbers vary in the microvasculature depending on the level of stress in the system), it may indeed be possible that inflammatory products do originate from them. However, it is difficult to distinguish the exact source of such inflammatory mediators. Dr. Fowler noted that these cells can be physically removed from the microvascular space, but to do so requires alteration of their glycoprotein surface. He also commented on the fact that this is a relatively new area of investigation and there is much to learn.

Dr. Assaad noted that arachidonic acid metabolites in instrumented sheep are generally fourfold higher than in uninstrumented sheep; thus, instrumented sheep may not be a good model. Dr. Glauser commented that his laboratory has also found elevated metabolites shortly after surgery in instrumented, intubated, anesthetized sheep; however, arachidonic acid metabolites are not elevated if experiments are done 4-5 days after surgical preparation in unanesthetized animals.

MAJ Ripple asked whether BAL can change lymph flow characteristics. Dr. Fowler responded that it does, but the changes are minimal. This would suggest that there are no great physiologic changes or injuries caused by the airway manipulation (due to the instrumentation). In conclusion, Dr. Fowler noted that one advantage of the sheep preparation is that approximately 75% of the lavage can be recovered, compared to only 40% in humans.

AGENTS WHICH MEDIATE PULMONARY EDEMA: ROLE OF ALVEOLAR MACROPHAGES

Dr. Caroline M. Kramer

The functional and biochemical responses of sheep alveolar macrophages can be measured in cells purified after BAL in animals exposed to inhalants which cause lung injury. This approach serves as a means to (a) identify affected cellular functions; (b) identify biochemical mechanisms of importance in the injury cascade; and (c) identify drugs which can modify the injury response.

In previous work, this system has been applied to studying the effects of inhaled soman in guinea pigs. Methods have now been established and validated in sheep. Male sheep weighing 30-40 kg were anesthetized by the intravenous administration of sodium thiopental (30 mg/kg). A flexible fiberoptic bronchoscope was inserted through a tracheostomy tube and gently wedged into a lower lobe bronchus. Five 50-ml aliquots of sterile 0.9% NaCl were slowly infused and returned by aspiration into the injecting syringe after a dwell time of less than 5 seconds. The recovered bronchoalveolar lavage (BAL) fluid represented greater than 60% of the original volume instilled and contained 25-30 x 10^6 cells. Differential counts performed immediately following the BAL showed 85% alveolar macrophages (AM), 11% lymphocytes, and 4% polymorphonuclear leukocytes with little erythrocyte contamination and >95% viability. Sepracell purification of the cellular fraction improved the differentials to >95% AM. Phagocytosis was measured by the internalization of opsonized chromated sheep erythrocytes over 180 minutes. By 90 minutes, phagocytosis had reached a plateau and remained threefold higher than baseline out to the 180-minute time point. Superoxide anion production by resting sheep AM (1.3$\pm$0.2 nM) was comparable to that found in the guinea pig AM. Stimulation of the response with phorbol-12,13-myristate acetate (PMA, 10^{-9}-10^{-6}M) resulted in a concentration-dependent increase in superoxide anion production with 10^{-6}M PMA being the optimal concentration.

These data indicate that bronchoalveolar lavage of sheep yields a functionally responsive population of AM that may be useful in furthering our understanding of sheep pulmonary function as well as interspecies differences.

AGENTS WHICH MEDIATE PULMONARY EDEMA: ROLE OF ALVEOLAR MACROPHAGES

Dr. Caroline M. Kramer, Dr. Richard A. Carchman, Fay K. Kessler, and R.B. Coles

ALVEOLAR MACROPHAGES FUNCTION AND BIOCHEMISTRY

SUMMARY

Sheep alveolar macrophage functional and biochemical responses will be measured in cells purified after bronchoalveolar lavage of naive or vesicant exposed sheep.

The goal is to provide a mechanism to:

1. Identify affected functions.
2. Identify biochemical mechanisms of importance.
3. Identify drugs or therapies which can modify the response.

EXPERIMENTAL APPROACH

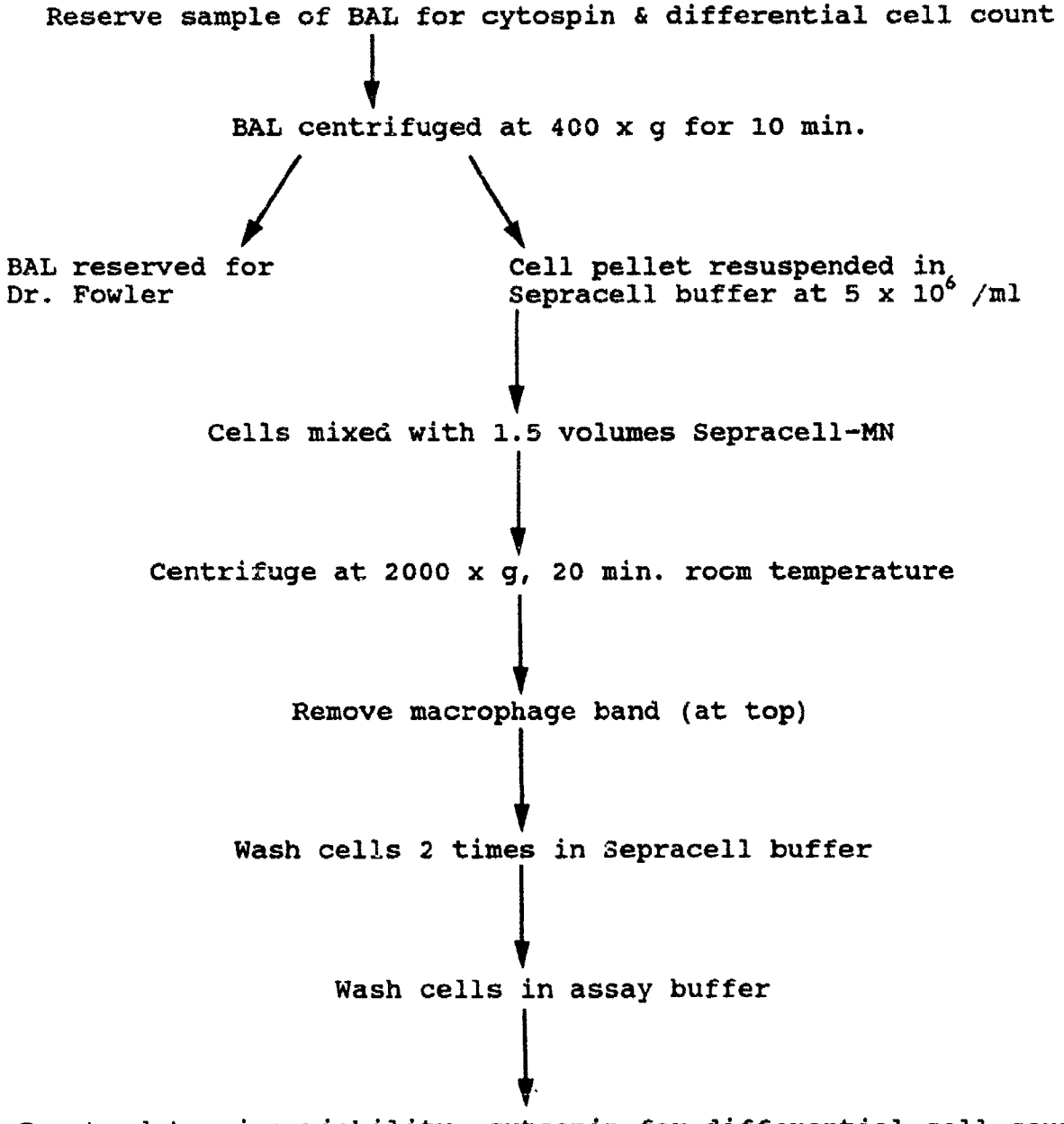
1. Sheep alveolar macrophage functional and biochemical responses will be measured *in vitro*.

2. Baseline and stimulated responses will be measured; stimuli will include FMLP, PMA, ionomycin.

3. Responses will be measured in naive and vesicant-exposed sheep.

SEPRACELL PURIFICATION

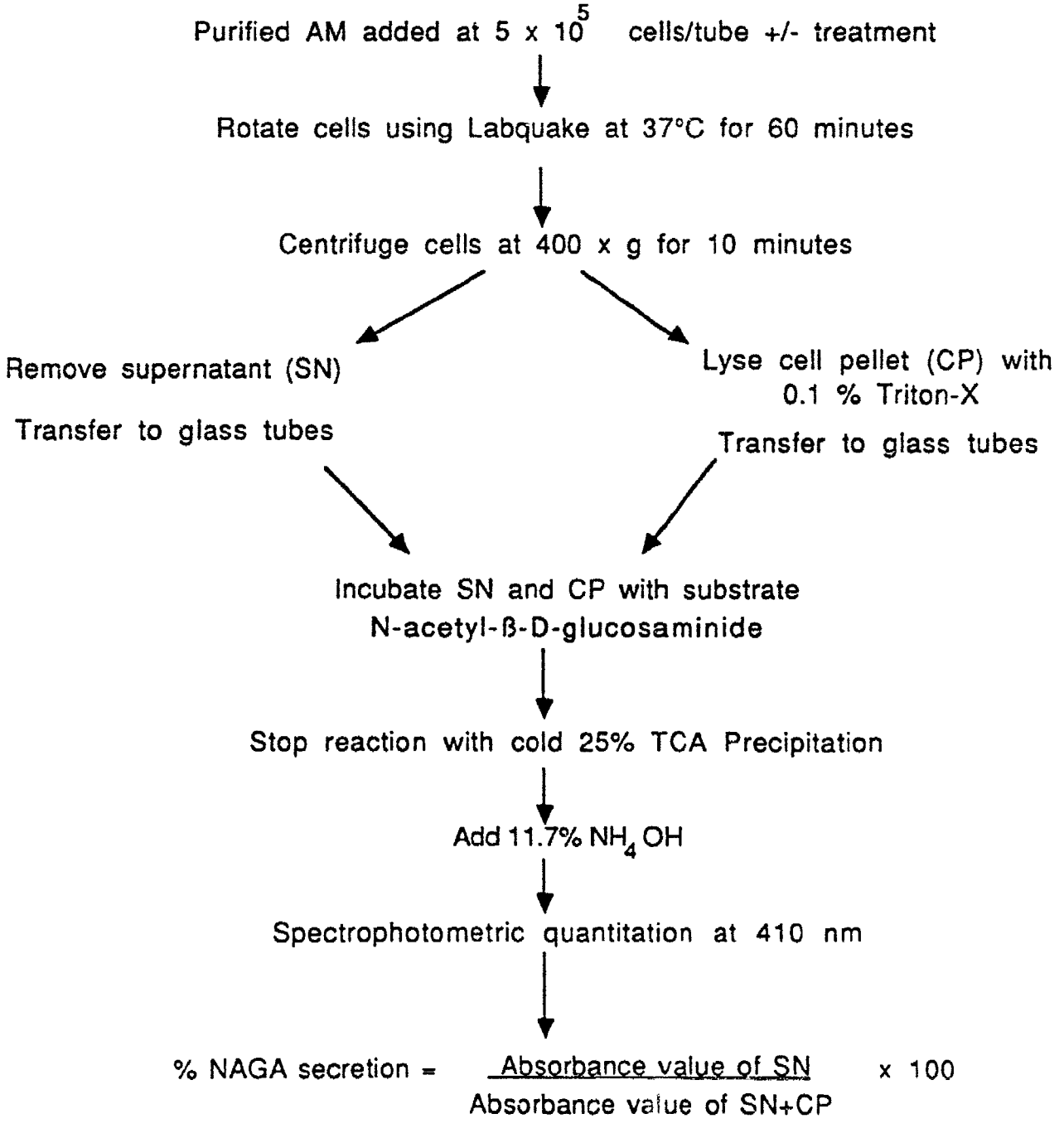
ALVEOLAR MACROPHAGES



	Cell yield	Differential cell counts		
		Lymphocytes	Monocytes	PMN's
Before Sepracell	29.4 x 10^6	9	89	2
After Sepracell	26.8 x 10^6	1	98	1

LYSOSOMAL ENZYME RELEASE

ALVEOLAR MACROPHAGES



ELASTASE ASSAY

ALVEOLAR MACROPHAGES

Purified AM added at 5×10^5 cells/tube +/- treatment

↓

Incubate at 37°C for 60 minutes

↓

Centrifuge cells at 400 x g for 10 minutes

↙ ↘

Remove supernatant (SN)

Transfer to glass tubes

Lyse cell pellet (CP) with 0.1 % Triton-X

Transfer to glass tubes

↘ ↙

Incubate SN and CP with substrate

methoxy-suc-ala-ala-pro-val-p-NA

↓

Stop reaction on ice

↓

Read absorbance at 410 nm

↓

$$\% \text{ elastase release} = \frac{\text{Absorbance value of SN}}{\text{Absorbance value of SN+CP}} \times 100$$

PHAGOCYTOSIS ASSAY

ALVEOLAR MACROPHAGES

Purified AM plated at 1 x 10^6 cells/well in 24 well TC plates

Adherence 90 minutes, 37°C, 5% CO_2

↓

Wash 3 times with DPBS

Replace with 0.5 ml DMEM + 10% FBS + 10 µl Ab-^{51}Cr-srbc

Incubate at 37°C, 5% CO_2 for desired time

↓

Check under microscope for rosetting

↓

Dump media into radioactive waste
Wash two times with warm DPBS

↓

Lyse cells with 1 ml 1 N NaOH, 5 min, 37°C

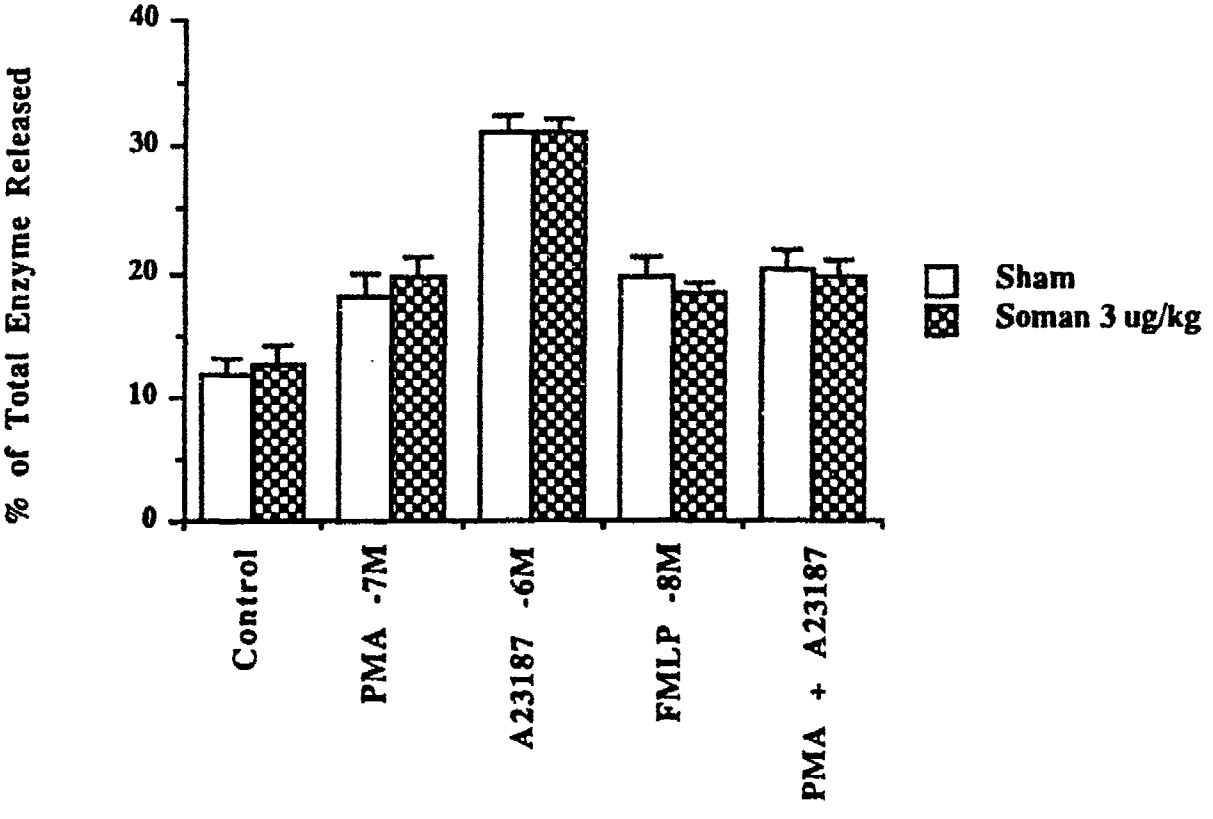
↓

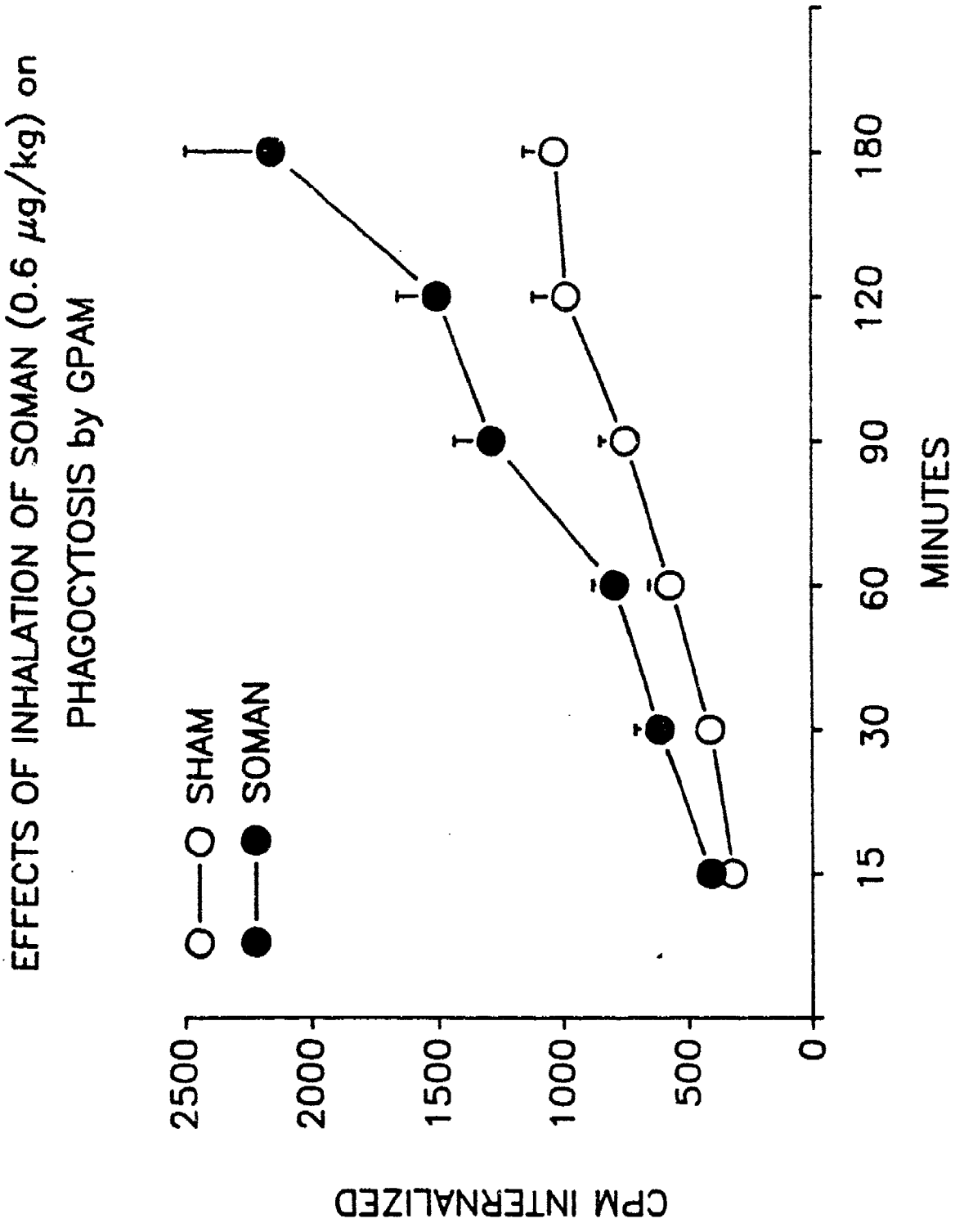
Determine cpm internalized by gamma counting

Calculate:

$$\frac{\text{cpm of experimental group}}{\text{cpm of control}} \times 100 = \% \text{ of control phagocytosis}$$

Soman Inhalation Effects on Lysosomal Enzyme Release




EFFECTS OF INHALATION OF SOMAN (0.6 μg/kg) on
PHAGOCYTOSIS by GPAM
O—O SHAM
●—● SOMAN
CPM INTERNALIZED
2500
2000
1500
1000
500
0
15
30
60
90
120
180
MINUTES

SUPEROXIDE ANION PRODUCTION

ALVEOLAR MACROPHAGES

Purified AM plated at 1 x 10^6 cells/well in 24 well TC plates

↓

Adherence 120 minutes at 37°C, 5% CO_2

↓

Wash cells once with RPMI 1640

↓

Wash cells with HBSS

↓

Add 80 µM cytochrome C in HBSS, +/- SOD
+/- stimuli

↓

120 minute incubation at 37°C

↓

Remove supernatant

↓

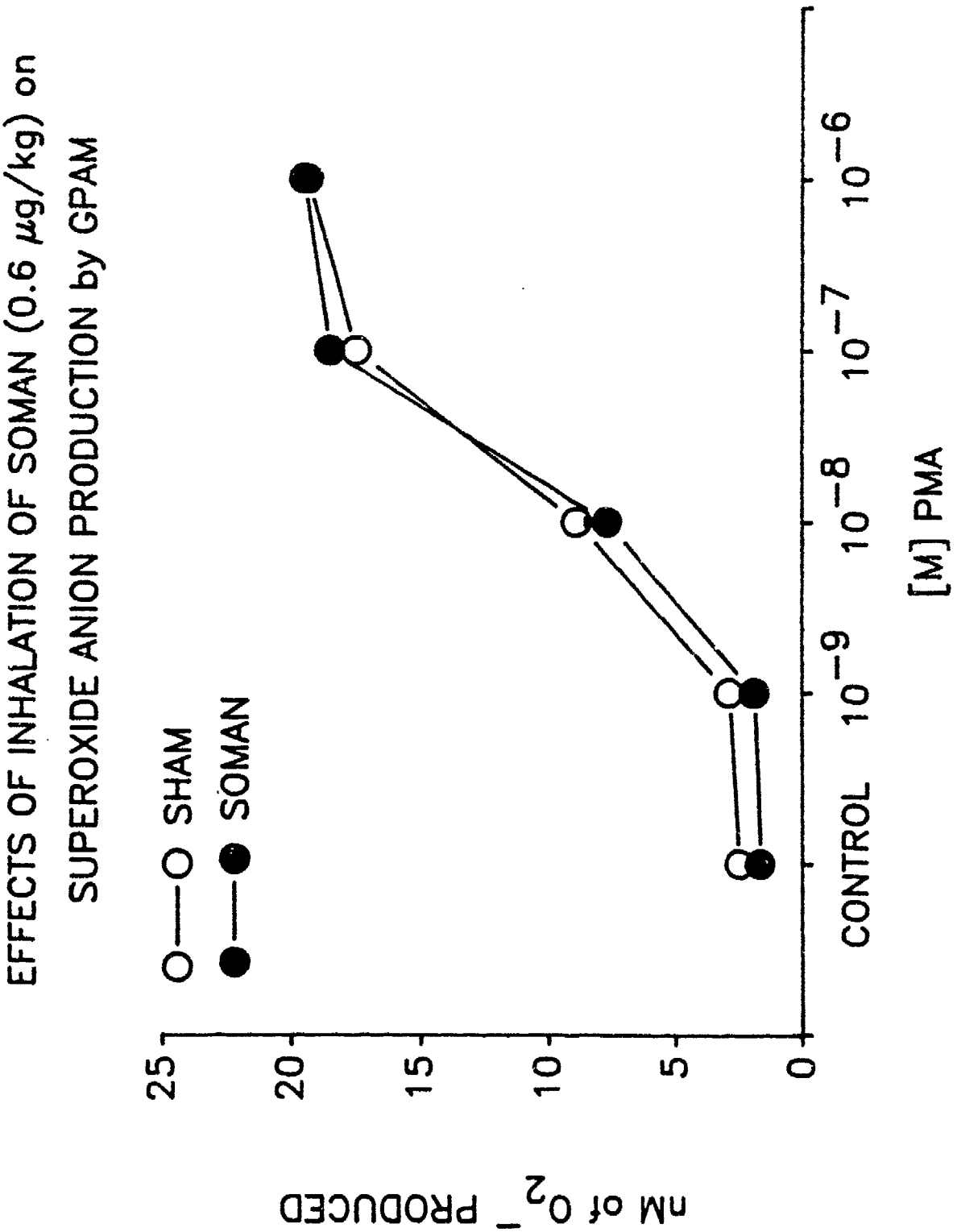
Centrifuge at 8000 x g for 2 minutes

↓

Absorbance at 550 nm determined

↓

nmoles superoxide anion = (Abs value - Abs (+SOD)) x 47.6


EFFECTS OF INHALATION OF SOMAN (0.6 μg/kg) on
SUPEROXIDE ANION PRODUCTION by GPAM
O—O SHAM
●—● SOMAN
nM of O_2^- PRODUCED
25
20
15
10
5
0
CONTROL
10^{-9}
10^{-8}
10^{-7}
10^{-6}
[M] PMA

CHEMOTAXIS ASSAY

ALVEOLAR MACROPHAGES

Chemoattractant added to blind well of Boyden chamber

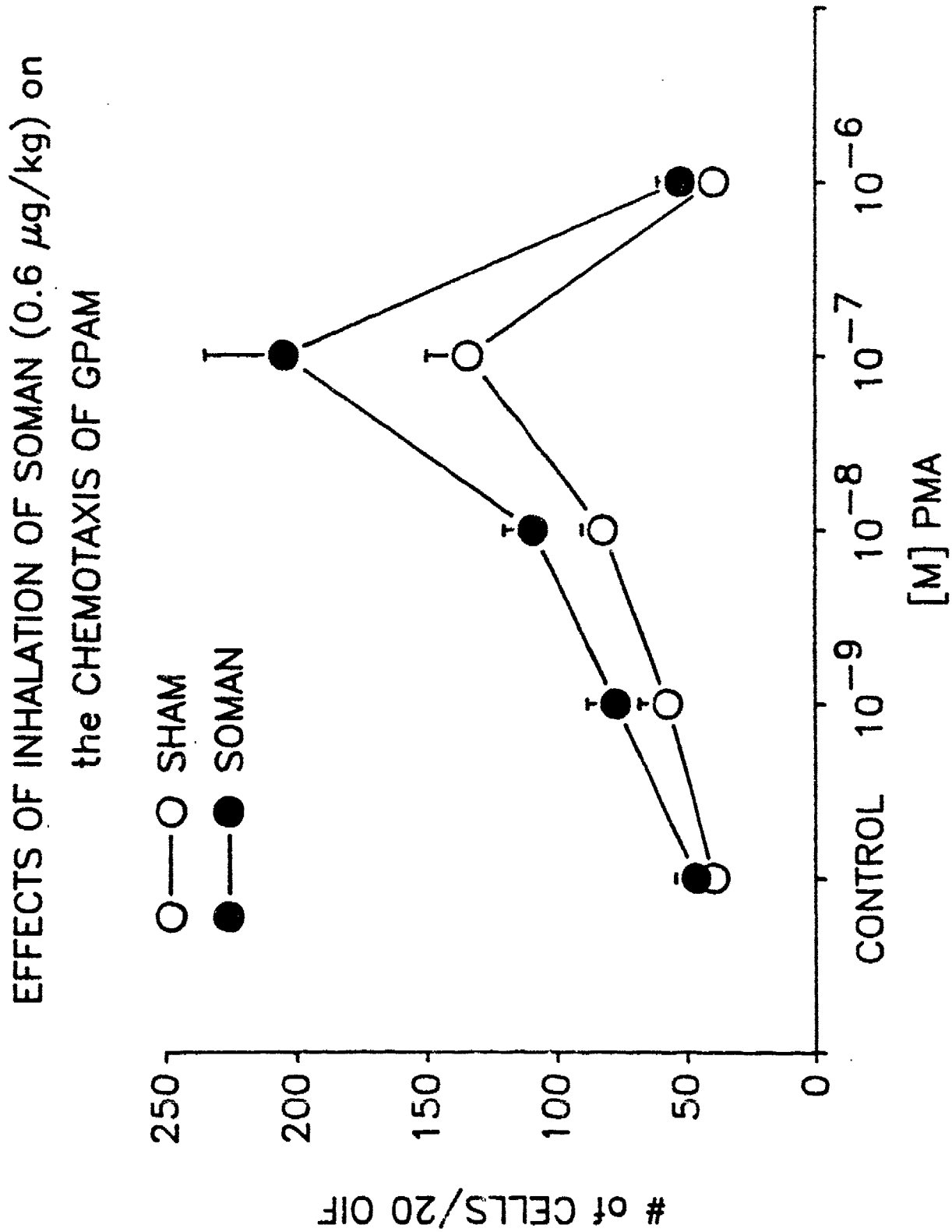
Polycarbonate filter (5 μ) placed over the blind well

Teflon nut tightened and 8×10^5 cells added to center well

Incubate 2 hours at 37°C

Remove and stain filter with Harleco Diff Quick

Chemotaxis is measured as the number of cells to migrate through the filter in 20 OIF


EFFECTS OF INHALATION OF SOMAN (0.6 μg/kg) on
the CHEMOTAXIS OF GPAM
O—O SHAM
●—● SOMAN
# of CELLS/20 OIF
250
200
150
100
50
0
CONTROL
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
[M] PMA

CYTOTOXICITY ASSAY

ALVEOLAR MACROPHAGES

P815 mouse mastocytoma cells and 3T3 mouse embryo fibroblasts

Label with ^{51}Cr

↓

1×10^4 cells/well plated in 96 well TC plates

Purified AM added to same wells in E:T ratios of 0.1:1, 1:1, 5:1, 10:1, and 25:1

↓

Centrifuge plates at 200 x g for 2 minutes

↓

Incubate for 24 hours at 37°C

↓

Centrifuge plates at 200 x g for 2 minutes

↓

Remove aliquots of supernatant

↓

Quantification of samples via Gamma counter

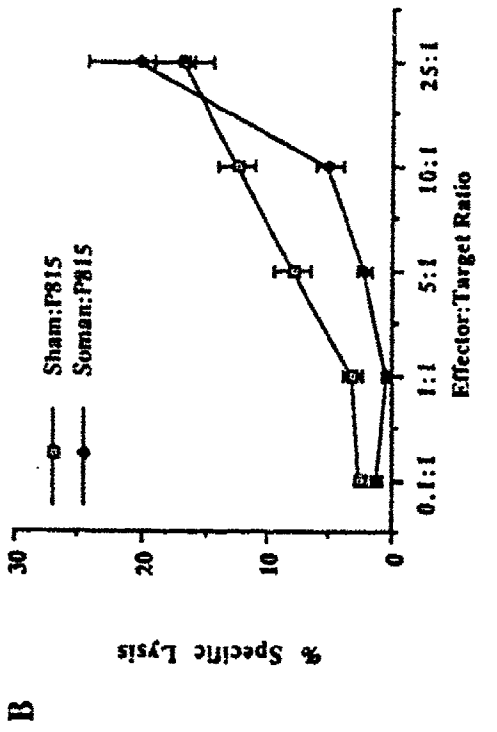
↓

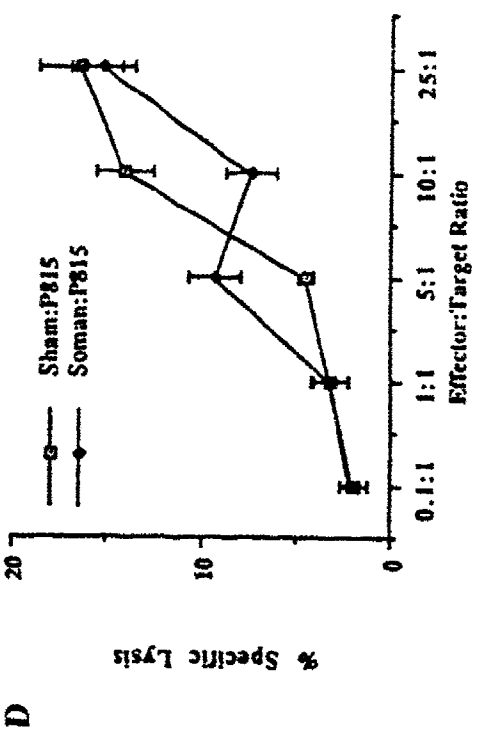
% specific lysis = [(A-B)/ (C-B)] X 100

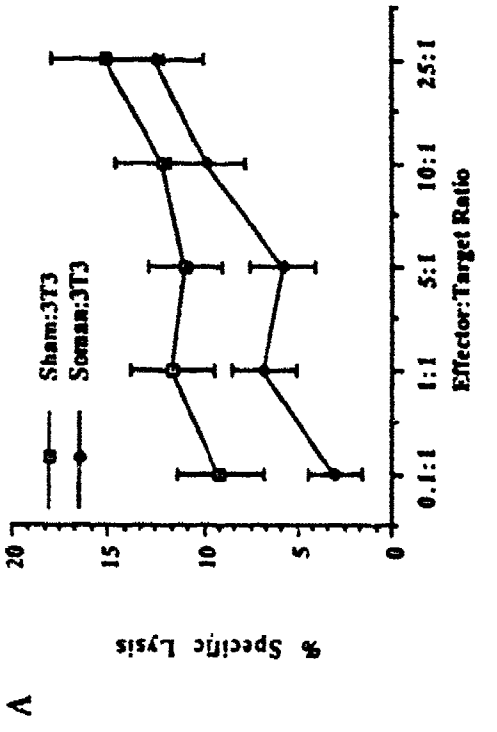
where A = cpm in experimental wells

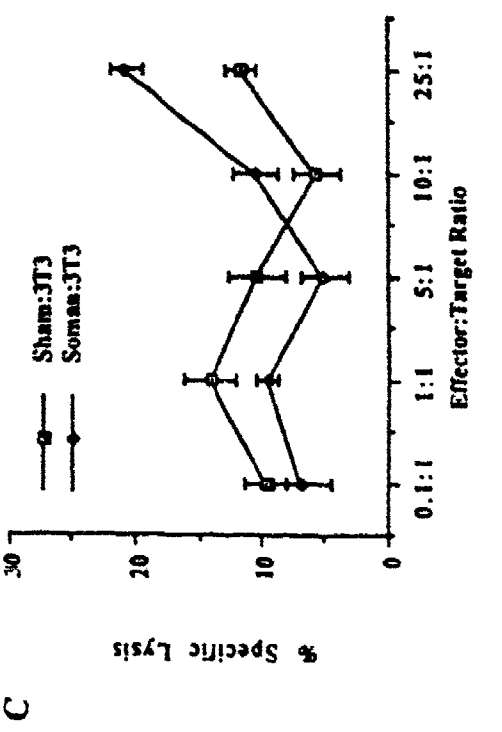
B = mean cpm released spontaneously

C = mean cpm for total incorporation/10^4 targets


B
% Specific Lysis
Sham:P815
Soman:P815
Effector:Target Ratio
0.1:1
1:1
5:1
10:1
25:1
0
10
20
30


D
% Specific Lysis
Sham:P815
Soman:P815
Effector:Target Ratio
0.1:1
1:1
5:1
10:1
25:1
0
10
20


A
% Specific Lysis
Sham:3T3
Soman:3T3
Effector:Target Ratio
0.1:1
1:1
5:1
10:1
25:1
0
5
10
15
20


C
% Specific Lysis
Sham:3T3
Soman:3T3
Effector:Target Ratio
0.1:1
1:1
5:1
10:1
25:1
0
10
20
30

MEASUREMENT OF CALCIUM CONCENTRATIONS USING FURA-2

ALVEOLAR MACROPHAGES

Purified AM washed and suspended in BSS with glucose at 10^6 cells/ml

↓

Cells loaded with 1 μM fura-2 methylester for 60 min.

↓

Cells aliquoted in microfuge tubes and placed on ice

↓

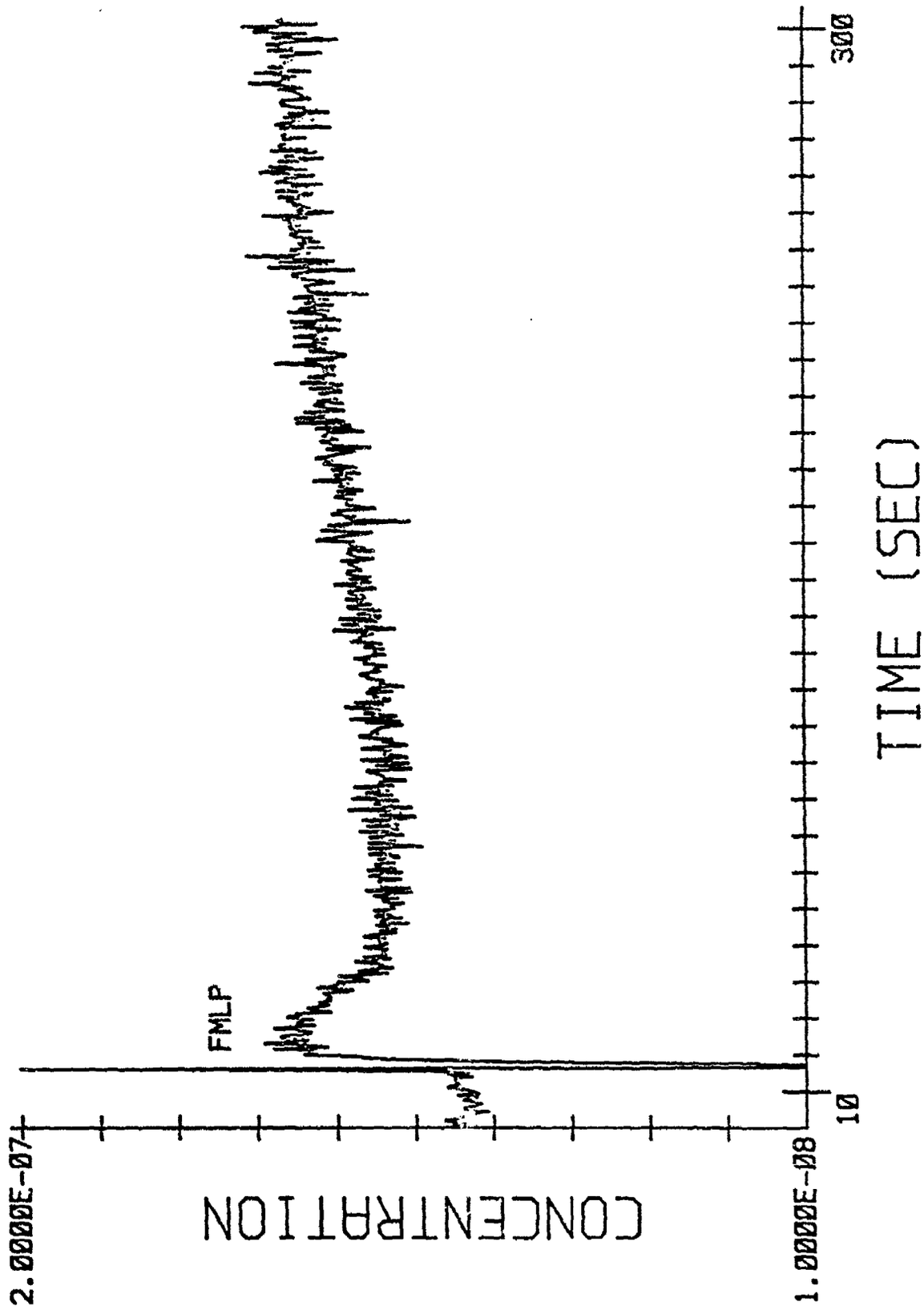
Aliquot centrifuged at 400 x g, 1 min., pellet resuspended in fresh warm BSS without fura-2

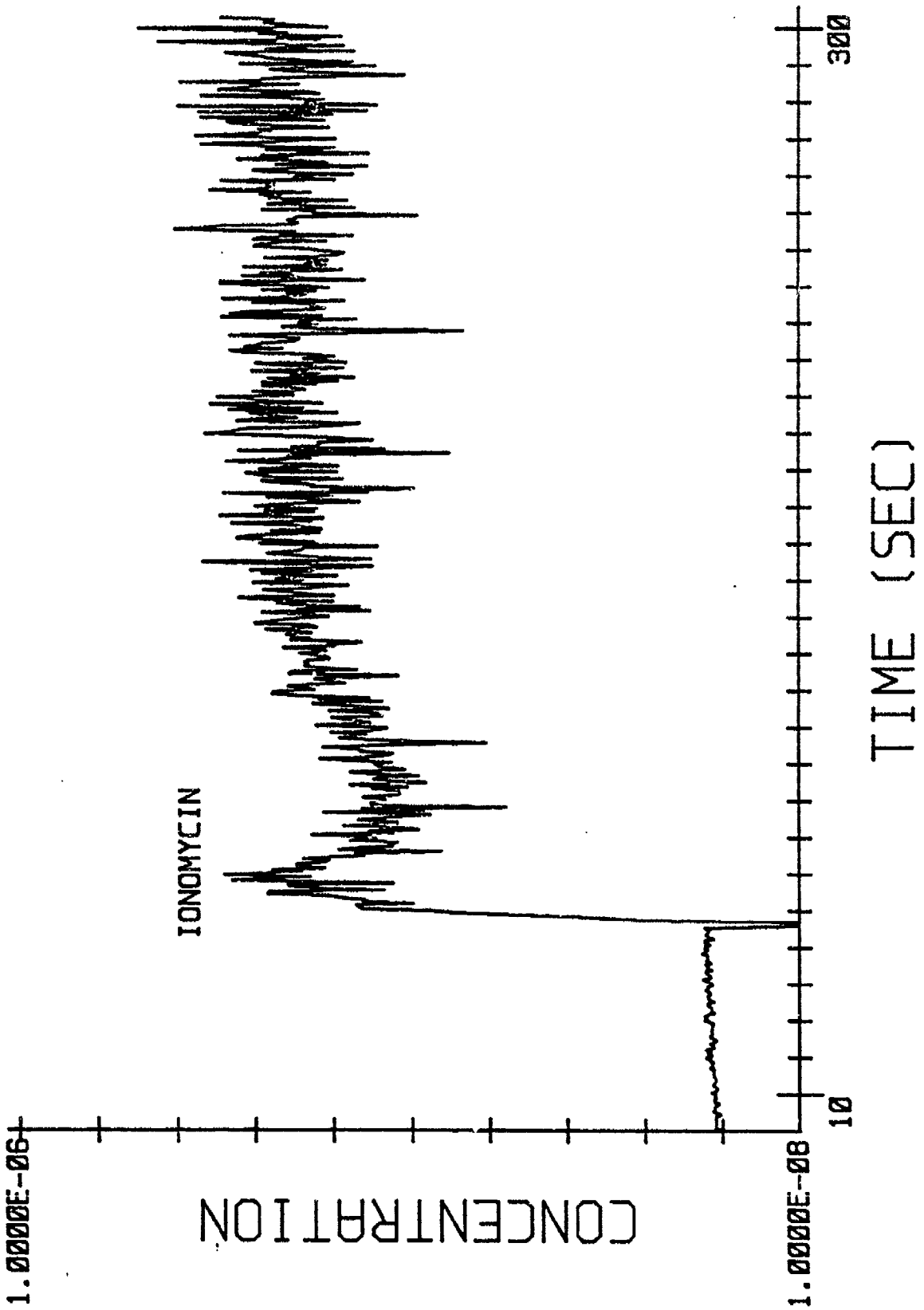
↓

Fluorescence is read simultaneously at 340 and 380 nm excitation, 550 nm emission, using SPEX dual beam spectrofluorometer

↓

Values converted to calcium concentrations using calibration curves


FMLP
2.0000E-07
1.0000E-08
CONCENTRATION
10
300
TIME (SEC)


IONOMYCIN
CONCENTRATION
1.0000E-06
1.0000E-08
10
300
TIME (SEC)

MEASUREMENT OF STEADY STATE CELL-ASSOCIATED ^{45}Ca ALVEOLAR MACROPHAGES

Purified cells adhered to 24 well TC plates
1 hour, 37°C, 5% CO_2 , in RPMI 1640

↓

Wash cells and replace with 1 ml/well fresh medium
containing 1 µCi ^{45}Ca

↓

Label to steady state (60 min.)

↓

Add stimulants over time course

↓

Stop reactions by removal of media

↓

Wash 3 times with warm RPMI

↓

Lyse cells with 0.5% SDS

↓

Determine cpm of cell-associated ^{45}Ca by scintillation counting

DISCUSSION AFTER DR. KRAMER'S PRESENTATION

LTC Keeler remarked that recent evidence indicated that tumor necrosis factor (TNF) played a role in this type of inhalant injury. Since alveolar macrophages are the main source of TNF, it would be a good idea to attempt to measure TNF in these experiments. Dr. Kramer agreed with this. However, it was noted by Dr. Glauser that there are no commercially available radioimmunoassays to measure sheep TNF. Dr. Assaad and Dr. Said noted that bioassays are available which can be used to measure TNF.

ISOLATED PERFUSED LUNG MODELS: SHEEP VERSUS RATS

Dr. Frederick L. Glauser

This presentation dealt with the advantages of the isolated perfused lung model for studying induced lung injuries, and compared the sheep and rat model systems. Among the advantages of this system is the fact that the perfusate can be well-controlled, with pressures and flow rates being carefully monitored. The system can be used to mimic the effects of exercise, and ventilation mechanics can be manipulated. It is also possible to measure endothelial and epithelial permeability in both directions using radiolabeled albumin, which can be added to the perfusate or directly into the alveolus.

However, the preparation of this model is technically difficult and unstable when using sheep; this is unfortunate since there are similarities in the respiratory physiology of sheep and humans. In contrast, the isolated, perfused rat lung model is technically easier, with high success rates obtainable.

ISOLATED PERFUSED LUNG MODELS: SHEEP VERSUS RATS

Dr. R.P. Fairman and
B. Smith-E-Incas

(Presented by Dr. Frederick L. Glauser

ISOLATED, PERFUSED SHEEP LUNG MODEL

- Advantages:

  Species similarities [between sheep and human respiratory physiology]

- Disadvantages:

  Expensive
  5-30% success rate
  Unstable
  Technically difficult

ISOLATED, PERFUSED RAT LUNG MODEL

- Advantages:

  Lab has extensive experience with this model
  > 90% success rate
  Stable
  Technically 'easy'
  Cheap
  Responds [somewhat] like sheep

- Disadvantages:

  Does not totally mimic the sheep model

DISCUSSION AFTER DR. GLAUSER'S PRESENTATION

Dr. Said asked why the isolated sheep lung preparation was so unstable. Dr. Glauser responded that the biggest problem is that most experimenters attempt to do this with sheep lungs *in situ*. In that preparation, it is necessary to get cannulas into the correct vessels and to flush a large volume of blood out very quickly--this is a difficult technique. Dr. Glauser also said that in his laboratory they see a continuing weight gain in the lungs, indicating that the *in situ* preparation is unstable. In contrast, in the rat model, the lungs are removed, and it becomes easier to manipulate the overall system. In addition, it is quite stable. Finally, Dr. Glauser commented that the rat model is well characterized, and where comparable data exist, it mimics the *in vivo* situation very well.

Concluding Remarks

CONCLUDING REMARKS
(Abridged)

MAJ David H. Moore

The formal presentations of this workshop are now concluded; however, much of our work is just beginning. In fact, we have some work ahead of us this afternoon addressing topics--mostly of a technical nature--introduced by the presenters here during the last two days. We have many details to cover that relate to safety issues, exposure systems, and the appropriateness of some of the proposed research. In addition, we want to talk about the in-house research that we have planned--for example, where do we begin with therapy? I encourage everyone here who is interested to attend that session.

I thank all of the presenters for the professional quality of their presentations, and I thank the participants and visitors for their contributions. Although an important session will take place this afternoon*, I will ask COL Dunn to make his concluding remarks now.

* The minutes of this session have not been included in this Proceedings.

CONCLUDING REMARKS
(Abridged)

COL Michael A. Dunn

I would like to thank our visitors for their presentations. I have seen and heard much excellent science over the last day and a half--from both our visitors and our in-house investigators. This meeting has come at an opportune time to enable all of us to share our efforts in this research area.

I am well aware of the problems encountered when a scientist attempts to secure a stable source of funding for research. Regarding this issue, I believe that the DoD recognizes the need for an ongoing research program directed toward solving the problems of toxicant-induced pulmonary injury.

Let us turn to the topic of the last couple of days--the organofluorines. The need for knowledge about mechanisms of action and potential avenues of therapy is critical. It is very much in the national interest to have high-quality, peer-reviewed papers published in the next year or so describing our progress in understanding organofluorine-induced lung injury and how close we are to developing valid therapeutic countermeasures to these agents. Making progress in this arena is urgent; I expect that MAJ Moore will structure the discussion this afternoon in terms of how we are going to get to there from here quickly.

It's a balancing act. On the one hand, we want to perform excellent science for science's sake, to keep those efforts well supported, and at the same time to maintain continuity in the research program. All of that is extremely important. On the other hand, we need to ensure that what we are doing is mission-relevant and will provide some means of attaining the Army's goals. Again, I urge you to structure your approach this afternoon to that end. For example, I did hear the argument for the use of the isolated, perfused rat lung model rather than the isolated sheep lung model: If you can show us how these studies can get us to where we want to go faster, more cheaply, and more effectively, you will get a receptive hearing. Again, thank you all very much.

Participants

PARTICIPANTS

Dr. Ayaad W. Assaad
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Suggested Reading

SUGGESTED READING

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