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Chemical Characterization and Toxicological Evaluation of Airborne Mixtures

Inhalation Toxicology of Diesel Fuel Obscurant Aerosol In Sprague-Dawley Rats

FINAL REPORT,
PHASE 1, ACUTE EXPOSURES

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Exposures of Sprague-Dawley rats to aerosolized diesel fuel in inhalation chambers are being conducted to determine the potential health effects of this visual obscurant. Data from the first phase of these exposures, establishment of maximum tolerated concentrations for single exposures, are reported here. Groups of rats of both sexes were exposed to concentrations of aerosol particles ranging from 2.7 to 16 mg/L for 2, 4, or 6 hours. Almost all deaths occurred within 48 hours of exposure. The only lesions observed at autopsy were in the respiratory tract: darkly reddened lungs, fluid in the trachea, and occasionally

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blood around the external nares. Mortality was highly correlated with the multiplication product of particulate concentration and duration of exposure ($p = 0.0001$) and 83% of the variation in mortality was explained by the Ct product, based on analysis using arcsine transformation. Assuming that effective dose was proportional to Ct, and using a log-linear-dose-response relationship, the maximum tolerated exposure condition, defined here as the lower confidence bound of the estimated one percent mortality level, was estimated to occur at a Ct product of 8 mg·hr/L for exposures of 2-6 hours.

Separate experiments were performed to establish dose-response relationships for two other endpoints: pulmonary free cell number after exposure and breathing pattern after exposure. After a single exposure to diesel fuel aerosol, the number of pulmonary free cells was found to have increased starting on the first day postexposure and continuing through the fourth day. On days one and two neutrophils were the predominant cell type, followed by a population shift to pulmonary alveolar macrophages on day four. Breathing frequency during exposure decreased linearly with increasing aerosol concentration in the range of 0.5 to 6 mg/L. For the diesel fuel aerosol, the RD50, defined as the concentration necessary to evoke a 50 percent reduction in respiratory rate, was estimated to be 3.75 mg/L.

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INHALATION TOXICOLOGY OF DIESEL FUEL OBSCURANT AEROSOL IN SPRAGUE-DAWLEY RATS

FINAL REPORT, PHASE 1, ACUTE EXPOSURES

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EXECUTIVE SUMMARY

Exposures of Sprague-Dawley rats to aerosolized diesel fuel in inhalation chambers are being conducted to determine the potential health effects of this visual obscurant. Data from the first phase of these exposures, establishment of maximum tolerated concentrations for single exposures, are reported here. Groups of rats of both sexes were exposed to concentrations of aerosol particles ranging in concentration from 2.7 to 16 mg/L for 2, 4, or 6 hours. Almost all deaths occurred within 48 hours of exposure. The only lesions observed at autopsy were in the respiratory tract: darkly reddened lungs, fluid in the trachea, and occasionally blood around the external nares. Mortality was highly correlated with the multiplication product of particulate concentration and duration of exposure ($p = 0.0001$) and 83% of the variation in mortality was explained by the Ct product, based on analysis using arcsine transformation. Assuming that effective dose was proportional to Ct, and using a log-linear dose-response relationship, the maximum tolerated exposure condition, defined here as the lower confidence bound of the estimated one percent mortality level, was estimated to occur at a Ct product of 8 mg.hr/L for exposures of 2-6 hours.

The original intent of this work was a mortality study to prepare for a series of repeat exposures, however, separate experiments were performed to establish dose-response relationships for two other endpoints: pulmonary free cell number after exposure and breathing pattern during exposure. These studies were limited to small numbers of animals and thus, although the data shows some very distinct trends, care has been taken not to use a battery of statistical analyses to interpret their meaning.

After a single exposure to diesel fuel aerosol, the number of pulmonary free cells was found to have increased, starting on the first day post exposure and continuing through the fourth day. On days one and two, neutrophils were the predominant cell type, followed by a population shift to pulmonary alveolar macrophages on day four.

Breathing frequency during exposure decreased proportionally with increasing aerosol concentration in the range of 0.5 to 6 mg/L for the diesel fuel aerosol. The RD50, defined as the concentration necessary to evoke a 50 percent decrease in respiratory rate, was estimated to be 3.75 mg/L.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Uses of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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INTRODUCTION

Battlefield smokes and obscurants are valuable tools of the armed forces for defending men, materiel, and installations against observation and bombardment. Because of their ability to degrade the performance of target acquisition and guidance devices, conceal friendly ground maneuver, deceive the enemy, and provide a means of signalling and marking, smokes and obscurants will be widely employed in the event of hostilities. The U.S. Army Medical Department, in cooperation with the U.S. Army Project Manager for Smokes and Obscurants, is actively investigating the toxic properties of various smoke/obscurant munitions and systems to estimate their potentials for adversely affecting the performance capabilities of soldiers in combat, for causing immediate or delayed health effects in troops exposed in training and for affecting the health, safety, and comfort of persons engaged in the manufacture of smoke munitions.

One material currently under study is diesel fuel aerosol. Aerosolized diesel fuel is a widely used visual obscurant. When injected into the exhaust manifold of a tactical vehicle, diesel fuel instantly vaporizes, is expelled with the vehicle exhaust, and upon exiting the exhaust system condenses to form a dense white "smoke" which rapidly provides a large and effective screen for the vehicle and supporting troops. Since there is a potentially large population at risk, and because little information is available on the potential health and performance effects of exposure to diesel fuel in this form, a number of studies have been designed to expand the available data base so that appropriate health protection decisions may be made by the U.S. Army Medical Department.

Inhalation exposures of rodents are being conducted to determine the biologic effects of exposure to varying aerosol concentrations, duration of each exposure, and frequency of exposures. The first step in these exposures was a series of acute, range-finding experiments to establish the maximum tolerated concentrations for a given exposure duration. The primary reason for conducting the acute exposures was to establish concentrations to be used in later repeated exposures. The exposure durations to be used in the repeated exposures were 2 and 6 hours. A duration of 4 hours was also used in the acute exposures to provide more complete information for statistical analysis.

Since exposure duration was a variable, we also attempted to gain whatever data were readily obtainable on the relation of mortality to the Ct product. The multiplication product of concentration (C) of an airborne contaminant and time of exposure (t) has often been used as an index of the "dose" of material delivered to the body and therefore the exposure conditions required for a specific effect (1), although this relation is not always valid and must be used with caution. The applicability of the Ct product to mortality could serve as a guide for its usefulness later in the repeated-exposure studies.

This report summarizes mortality data from these acute exposures and from additional acute exposures which were performed to establish a concentration-response relation for two other endpoints: pulmonary free

cell number after exposure and breathing pattern during exposure. The next phase involves multiple exposure of 12 groups of animals with varying aerosol concentration, exposure duration, and frequency. The last phase is of similar design but longer duration. Results from these repeated exposures will be reported at a later date.

Only the biologic aspects of the acute exposures are dealt with here. The chemical and physical aspects of aerosol generation and monitoring are described elsewhere (2-4). It should be noted that "concentration" refers to the particulate phase of a condensation aerosol. Depending on aerosol concentration, as much as 20 percent of the originally vaporized diesel fuel may not condense into particles and will remain as a vapor phase at the concentrations and chamber temperatures in this phase of the study.

MATERIALS AND METHODS

MORTALITY AFTER SINGLE EXPOSURES

Sprague-Dawley rats of both sexes were used for all exposures. Animals were obtained commercially (Charles River, Wilmington, Massachusetts) and quarantined for two weeks after arrival at our facility. Those animals shedding Pseudomonas during quarantine were not brought to the inhalation facility. No Sendi or mycoplasma were observed in the animals selected for testing from each shipment.

Once in the inhalation facility, rats were housed individually in hanging, stainless steel, wire mesh cages. Purina rat chow was provided ad libitum except during exposures. Water was provided using an automatic watering system. In order to control the possible presence of Pseudomonas aeruginosa the water supply was hyperchlorinated to 16 ppm as it entered the building. The actual chlorine concentration in the water the animals received was in the range of 3-5 ppm; a concentration range that is commonly used in animal facilities to prevent the spread of the bacteria. A 12 hr-on/12 hr-off light cycle was maintained; most exposures were begun in the morning. The age at exposure varied from 12 to 15 weeks. All rats were observed for at least 2 weeks after exposure.

The following experimental design was developed to characterize the relationship of acute exposures to mortality. Specific objectives were (1) to estimate the exposure conditions at which minimal (e.g. 1%) mortality would occur and (2) to ascertain if mortality could be adequately modeled as a function of the Ct product. For example, the proportion (or some transformation thereof), Y, of individuals dying would be modeled as

$$Y = \beta_0 + \beta_1 \log (Ct) + \text{error}$$

where β_0 and β_1 are parameters fitted to the data. This model would be assessed in terms of its ability to explain the observed responses, as compared to more general models such as

$$Y = \beta_0 + \beta_1 \log (Ct) + \beta_2 \log t + \text{error}$$

A model deemed as providing a good fit would then be used to determine the harshest treatment or treatment combinations at which a reasonably low mortality could still be expected. The combinations chosen and the number of replications of some combinations of C and t were based on considerations of symmetry and the aim to estimate the nature of the response surface. Ten animals (5 males and 5 females) were used in each exposure.

The highest concentration ever used was 16 mg/L because of mortality and the threat of explosion. With aerosol concentrations of 55-58 mg/L we observed explosions in 9 or 14 trials (64%) in a small container with an electric arc. With concentrations of 50-54 mg/L, 5 of 19 experiments (26%) resulted in explosions. At concentrations below 50 mg/L, we observed no explosions.

A single lot of reference grade fuel (Diesel Fuel type 2-D as specified in the Code of Federal Regulations, Title 45, Subtitle A, Part 1201, Subpart J, paragraph 120.121), obtained from Phillips Petroleum Company, was selected as the source of smoke aerosol for these studies. This fuel was not immediately available, and preliminary range finding studies were conducted using diesel fuel obtained from a local pumping station (commercial fuel). The toxic responses to the two diesel fuels were dissimilar. The results from acute exposures to both sources of diesel fuel are presented to illustrate the variability in biologic response that can occur with fuel from different sources. For all subsequent studies, the reference grade fuel was used to reduce this source of variability and avoid confounding of comparisons among the experimental parameters.

The Phillips fuel was stored in refrigerated 55 gal drums. As batches of fuel were required, the drums were warmed, tumbled, and split into 5 gal portions. These 5 gal containers were used to supply reservoirs on the aerosol generators during exposure.

The order of the exposures with either fuel was randomized within blocks of eight each. This allowed the use of information from initial blocks to aid in the choice of points to replicate in subsequent blocks. The exposure sequence in each block was randomized in order to guard against any systematic bias introduced by different groups of animals and minor changes in the aerosol. Two blocks of exposure to commercial fuel were completed before the unavailability of rats forced a delay. The third block of exposures to commercial fuel was done 1.5 months later. The same sample of commercial fuel was used, having been stored in the dark at room temperature. There appeared to be a disparity between the results in the first two blocks of exposures and the third in that toxicity was markedly decreased during the later exposures (see Results, p. 11). To determine if this change in apparent toxicity could be traceable to some minor changes in the design of the aerosol generator, additional exposures were run at

2.7 mg/L for 6 hours (a highly toxic exposure initially) with both the old and new style of generator. There did not appear to be any relation between the style of generator and mortality.

ALTERATIONS IN PULMONARY FREE CELLS AFTER A SINGLE EXPOSURE

One of the endpoints being investigated after repeated exposures is the number of pulmonary free cells which can be recovered by tracheal lavage and the phagocytic activity of the lavaged alveolar macrophages. It was therefore of interest to see if these parameters could be altered by a single exposure to aerosolized diesel fuel. To this end, a group of female rats was exposed to 4 mg/L of diesel fuel for 2 hours (a Ct of 8). Three exposed animals plus 2 untreated controls were killed at 1, 2, 3, 4, and 7 days later by pentobarbitol anesthesia and aortic exsanguination. Previous work in this laboratory has shown that there is a relationship between the body weight of an animal and its vital capacity. This relationship was used and the lungs were lavaged 6 times with a volume of saline that was 4 mL less than the animals theoretical vital capacity. The washings were combined and the lavaged cells were centrifuged at 300g for 10 minutes, the supernatant removed and the cells resuspended in saline. This procedure was repeated a second time to thoroughly rinse the cells. After the third centrifugation cells were resuspended in Hank's balanced salt solution.

An aliquot of this cell suspension was taken for determination of total and viable (as determined by trypan blue exclusion) cells and alveolar macrophages. These counts were made on a hemocytometer. Another portion of the cell suspension was diluted to give 2×10^5 viable cells/mL and was used in a phagocytosis assay. Cells were allowed to settle and attach on a glass coverslip while incubated with yeast for 1 hour. The cells which attached to the coverslips were subsequently washed to remove excess yeast, fixed with buffered formaldehyde, stained with Wright's stain, and examined for cell type and yeast uptake.

BREATHING PATTERN DURING EXPOSURE

Breathing frequency and minute volume (the product of breathing frequency and the volume per breath or tidal volume) often decrease in response to the sensory irritancy of inhaled gases and aerosols (5, 6). These parameters were measured during exposure of animals to aerosolized diesel fuel to gain more information on its irritant potential. Exposures were performed with rats restrained in nose-only tubes which could be used as body plethysmographs. Thus absolute measurements of breathing frequency and tidal volume were performed. Subsequently, frequency and relative tidal volume were recorded during whole-body exposure of unrestrained animals for comparison, a less defined method but more related to the true exposure situation.

To measure breathing pattern during nose-only exposure to diesel fuel aerosol, rats were restrained in solid plexiglass tubes (A) shown in

Figure 1. The tubes had a cone on the inside of one end into which the animal's head was drawn. The cone had previously been fitted with a wax mold to fit a given size of animal. A paper clip was inserted through the hole in the end of the cone, fastened around the rat's teeth, drawn back to pull the rat's head securely into the wax mold, and bent to retain the rat in position. Silicone grease was used to seal around the rat's head. The rat's body was left free to move in the tube. The tubes containing the rats were then inserted into the side of a 350 mL plexiglass chamber (B) so that the rats' noses protruded into the chamber interior. Aerosolized diesel fuel was drawn through this small chamber from a larger whole-body exposure chamber as shown in the figure.

Breathing frequency and total volume were determined by making the rat tubes into body plethysmographs. A rubber stopper sealed the open end except for tubing connecting the rat tube to a 3 L metal container. Pressure fluctua-tions from the rat's breathing were measured by a Validyne MP45 differential pressure transducer (C). A constant reference pressure was established with a 5 L copper-filled flask. Pressure was initially equilibrated across the transducer by opening stopcocks (D). Calibrations were performed by injecting known volumes of air into the plethysmograph with a rat in place.

Exposures lasted two hours. Breathing pattern was recorded before exposure began and then at 5, 10, 15, 20, 60, 90, and 120 minutes after the start of exposure. There were 4 rats for each combination of sex and concentration, except for sham-exposed controls which consisted of 8 animals per sex.

Whole body exposures were performed on females only, eight per group. Each rat was placed in a Mason jar, as shown in Figure 2. Aerosol was drawn from a central manifold on the side of the larger exposure chamber simultaneously through each of 8 jars at 1 Lpm. Gravimetric determinations of particulate concentrations were made directly from samples taken from the Mason jars. Breathing pattern was recorded using a barometric technique (7). The two valves into the jar were closed. Air in the jar was warmed and humidified during inhalation by the rat so that thoracic expansion was greater than the volume of air inhaled from the jar. The net effect was an increase in pressure in the jar, as measured by the differential pressure transducer. Breathing frequency was recorded easily, but tidal volume was not readily calibrated because of complications from the presence of aerosol. Thus only relative tidal volume was obtained.

RESULTS

MORTALITY AFTER SINGLE EXPOSURES

Mortality in the first two blocks of acute exposures (commercial fuel) is summarized in Table 1. Almost all deaths occurred within 48 hours after exposure and generally during the night after exposure. The only lesions

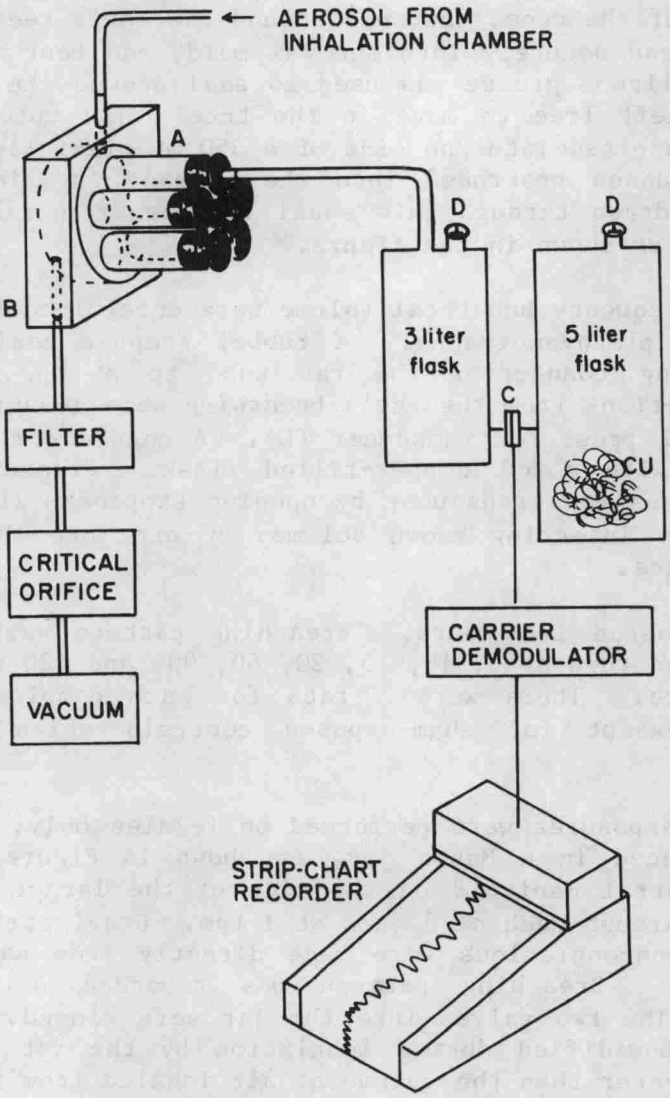

Figure 1

Schematic diagram of apparatus used for recording breathing pattern of rats during nose-only exposure to aerosol. Note restraint tubes for rats (A), 350 mL chamber (B), differential pressure transducer (C), and 3-way valves (D).

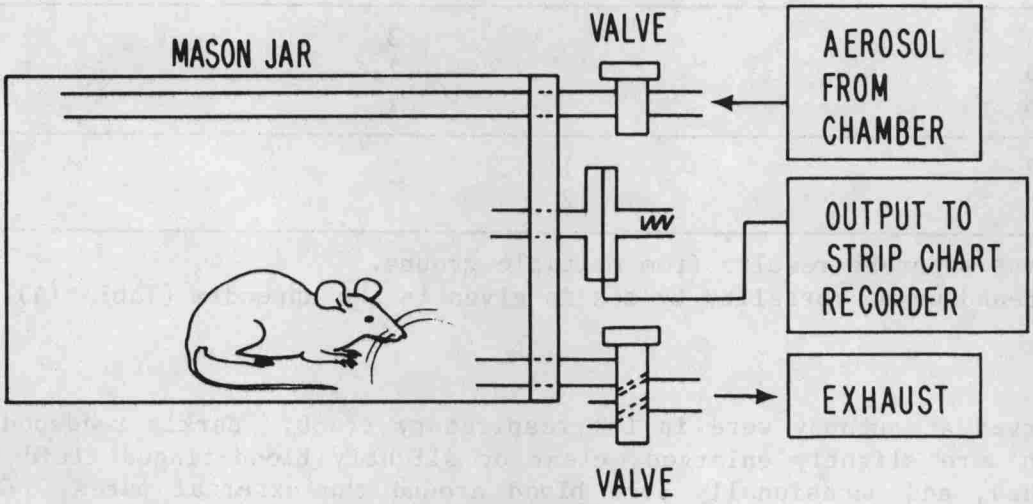

Figure 2

Schematic diagram of apparatus used to record breathing of rats during whole-body exposure to aerosol.

TABLE 1. MORTALITY AMONG GROUPS OF 10 RATS AFTER SINGLE EXPOSURES IN FIRST TWO BLOCKS OF EXPOSURES TO COMMERCIAL FUEL AEROSOL

Concentration (mg/L)	Hours of Exposure			
	1	2	4	6
0.7				0
1				
1.3				1,2[a]
2			3,3	5
2.7				10
3			8,9	
4		3	9	
6		1,6		
8		1		
12	1			
16	2			

[a]Commas separate results from multiple groups.
A breakdown of mortality by sex is given in the appendix (Table 1A).

observed at autopsy were in the respiratory tract: darkly reddened lungs which were slightly enlarged, clear or slightly blood-tinged fluid in the trachea, and occasionally some blood around the external nares. Grossly observable changes were limited to labored breathing in a relatively few animals.

The mortality from the first two blocks is expressed in relation to the Ct product in the appendix (Table 2A). It is readily apparent that the variability for the mortality at a given Ct product is quite large and there was no obvious relationship between mortality and Ct product. After a delay of approximately 45 days caused by the non-availability of animals the third block of exposures was started to fulfill the experimental design.

As seen in Table 2, mortality in the third block of exposures was less than might be expected from the results of the previous exposures (exposure to 2.7 mg/L for 6 hours was not originally part of the third block). Some minor changes had been made in the generator during the 45 day delay and there was some concern that these changes might have resulted in decreased mortality. Therefore a comparison was made between old and new style generators under exposure conditions which had previously caused 100 percent deaths (2.7 mg/L for 6 hours). Mortalities were 0/10 and 1/10 for the new style generator and, with the old style generator, 2/10 and 6/10. It was concluded that the decrease in the mortality in the third block

compared with the first two blocks was not due to any changes in the aerosol generator. This conclusion is based on:

1) use of the old style generator did not result in 100 percent mortality as it had previously.

2) the range in mortality from the use of two slightly different generators was no greater than that observed within the first two blocks of exposure (viz. 6 mg/L for 2 hours).

At this time the Phillips reference grade fuel became available and this matter was not pursued any further.

Mortality after exposure to an aerosol of Phillips fuel appeared to be decreased in comparison to the previous exposures with locally obtained commercial fuel. However, as before, all deaths occurred within 48 hours of exposure. There were no delayed deaths during two weeks of observation. Gross observations during autopsy were identical to those after exposure to commercial fuel.

Mortality during 3 blocks of exposures to Phillips fuel is summarized in Table 3 and Figure 3. There was no sex difference in mortality so data on both sexes were pooled. Statistical analysis of the mortality data began with exploratory regressions of the proportions dying (and various transformations thereof, including probit, logit and arcsine) on the factors defining the treatment groups [i.e., concentration (C), time (t), and "dose" (Ct)]. In general, the arcsine transformation provided the best

TABLE 2. MORTALITY AMONG GROUPS OF 10 RATS AFTER SINGLE EXPOSURES IN LAST BLOCK OF EXPOSURES TO COMMERCIAL FUEL AEROSOL

Concentration (mg/L)	Hours of Exposure			
	1	2	4	6
0.7				0
1			0	
1.3				
2		0,0		
2.7				0,1,2,6
3				
4		0		
6		0		
8		0		
12				
16				

A breakdown of mortality by sex is given in the appendix (Table 3A).

fit and the p-values given here are for analyses using that transformation. It was found that

1) mortality was highly correlated with the Ct product ($p = 0.0001$) and 83 percent of the variation in mortality was explained by Ct,

2) the response and transformations of the response seemed more nearly linear in ln (Ct) than in Ct,

3) the linear model involving ln (Ct) [response = $\beta_0 + \beta_1 \ln (Ct) +$ error] is essentially as good as the model involving both ln C and ln t [response = $\beta_0 + \beta_1 \ln C + \beta_2 \ln t +$ error]. Incorporating t into the model improved the explained variation to 91 percent. That is, the following model was fitted: response = $\beta_0 (t) + \beta_1 (t) \ln (Ct) +$ error, where $\beta_0 (t)$ and $\beta_1 (t)$ denote separately fitted intercepts and slopes for different values of t. This improvement was associated with a slight shift in the intercepts of the response lines ($p = 0.88$). The Ct-response line for 2 hour exposures was slightly above the other two; however, the line for 4 hour exposures was lowest. There was no clear ordered relation of response to exposure time (see Figure 4).

By acceptance of the hypothesis that mortality is roughly a function of the Ct product, the design questions for repeated exposures became fairly straightforward to answer. A Ct product of 8 was calculated by probit analysis as the lower confidence bound for the Ct product expected to result in 1 percent mortality. Thus exposure to a Ct of 8, or slightly higher levels, was taken as an estimate of a maximum tolerated "dose", with the stipulation that the actual dose of particles retained in the body was undefined.

TABLE 3. MORTALITY AMONG GROUPS OF 10 RATS AFTER SINGLE EXPOSURES TO AEROSOLIZED PHILLIPS DIESEL FUEL

Concentration (mg/L)	Hours of Exposure		
	2	4	6
2.7			0
4		0	3
5.3			3
6		0,0	
8	0,1	4	6
12	2	6	10
14	5		
16	5	6	10

A breakdown of mortality by sex is given in the appendix (Table 4A).

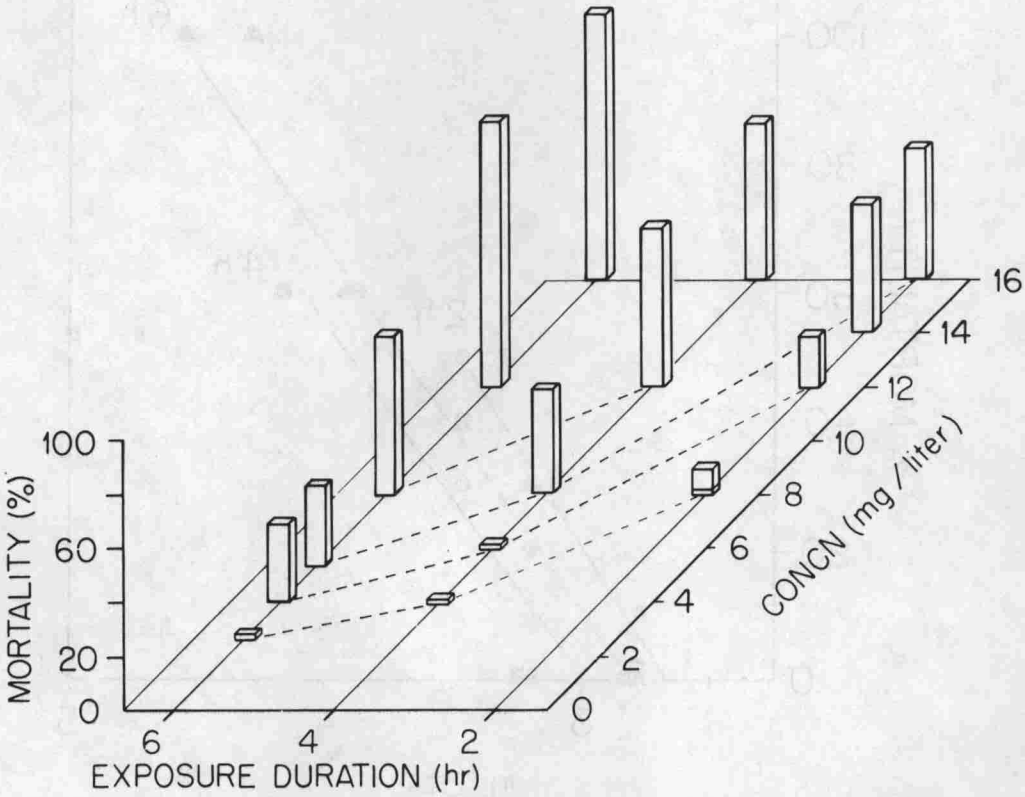

Figure 3

Three-dimensional graph of mortality among groups of rats after single exposures to varying combinations of aerosol concentration and exposure duration. Dashed lines connect points of equivalent Ct products.

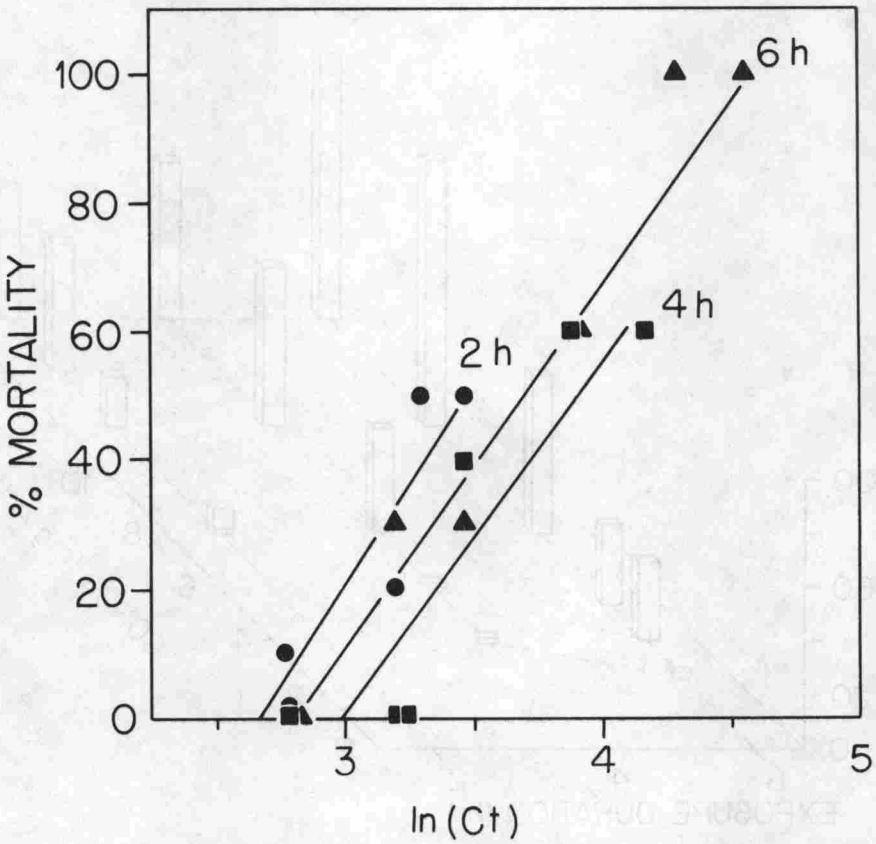

Figure 4

Relation of mortality after single aerosol exposures to ln (Ct) for exposures of 2 (●), 4 (■), or 6 (▲) hours of duration.

It has been our experience in the repeated exposures of rats to diesel fuel aerosol that the prediction of mortality from this acute study was accurate. Repeated exposures (up to 9) to a Ct of 8 resulted in no mortality, while some deaths have occurred with a Ct of 12.

ALTERATIONS IN PULMONARY FREE CELLS

Counts of total and viable (determined by trypan blue exclusion) cells and alveolar macrophages after a single exposure to 4 mg/L for 2 hours were carried out using a hemacytometer. The cell counts were normalized to body weight in order that comparisons between this series of experiments and subsequent experiments using rats of both sexes and of different ages could be made with greater facility. The results are summarized in Table 4. Almost all cells lavaged from untreated animals were identified as macrophages. Total free cells increased markedly at days 1 and 2 after exposure and then slowly declined to pre-exposure values by day 7. The number of alveolar macrophages appeared to decrease shortly after exposure, increase by day 4 to about 90 percent of the increased number of lavaged cells, and return to control values by day 7.

TABLE 4. MILLIONS OF LAVAGED PULMONARY FREE CELLS AT INTERVALS AFTER A 2 HOUR EXPOSURE TO 4 mg/L OF AEROSOLIZED DIESEL FUEL (MEAN ± S.E.)

	Total cells	Alveolar macrophages
Days post-exposure	Kg body weight	Kg body weight
Control	10.2 ± 2.3	9.0 ± 2.2
1	24.5 ± 7.7	5.4 ± 2.5
2	23.9 ± 2.0	6.4 ± 0.5
3	22.2 ± 2.6	9.9 ± 0.9
4	20.8 ± 6.0	17.5 ± 5.1
7	10.1 ± 3.5	No data

A comparison of the percent of the cells which were macrophages is given below for 1) viable cells counted on hemocytometer before incubation on the coverslip and 2) cells attached to coverslip after incubation (Table 5). The cell counts by the two methods agreed very closely. Therefore there was no evidence that selective attachment of macrophages to the coverslip occurred or that cell identification on the hemocytometer was in error.

TABLE 5. MACROPHAGES AS PERCENT OF TOTAL VIABLE LAVAGED CELLS OBSERVED ON HEMOCYTOMETERS COMPARED WITH MACROPHAGES AS A PERCENT OF TOTAL CELLS OBSERVED ON COVERSLIPS

Day post-exposure	Hemocytometer	Coverslip
Control	95.4%	97.3%
1	17.0	15.3
2	26.6	23.0
3	42.1	42.6
4	89.0	92.3
7	No data	97.9

The relative compositions of attached cell populations after exposure are shown in Figure 5. There was a rapid increase in the neutrophil population by day 1 post exposure which remained highly elevated through day 3. The neutrophil population was still elevated approximately threefold above preexposure levels on day 4. The absolute number of lavaged alveolar macrophages decreased through day 2 and was markedly elevated above control values on day 4 (Table 4). The binding of yeast by the lavaged macrophages was not affected by exposure to diesel fuel.

If the population of lavaged cells changed after one exposure to 4 mg/L, is there a dose-response to concentrations over the range used in our experiments? Groups of 4 male and 4 female rats were exposed for 2 hours to 0, 1.3, 2, 4, or 6 mg/L and their lungs lavaged 2 days later, the time of maximum increase in cell number after 4 mg/L. The numbers of total cells and alveolar macrophages are summarized in Table 6.

TABLE 6. MILLIONS OF LAVAGED PULMONARY FREE CELLS AT 48 HOURS AFTER A 2 HOUR EXPOSURE TO VARIOUS CONCENTRATIONS OF AEROSOLIZED DIESEL FUEL (MEAN ± S.E.)

	Total Cells	Alveolar macrophages
mg/L	Kg body weight	Kg body weight
Control	13.6 ± 2.4	10.0 ± 1.3
1.3	16.4 ± 1.5	11.5 ± 1.1
2	16.8 ± 3.1	8.2 ± 1.4
4	26.4 ± 6.5	4.8 ± 1.4
6	26.3 ± 3.2	4.1 ± 0.7

A breakdown of these results by sex is given in the appendix (Table 5A).

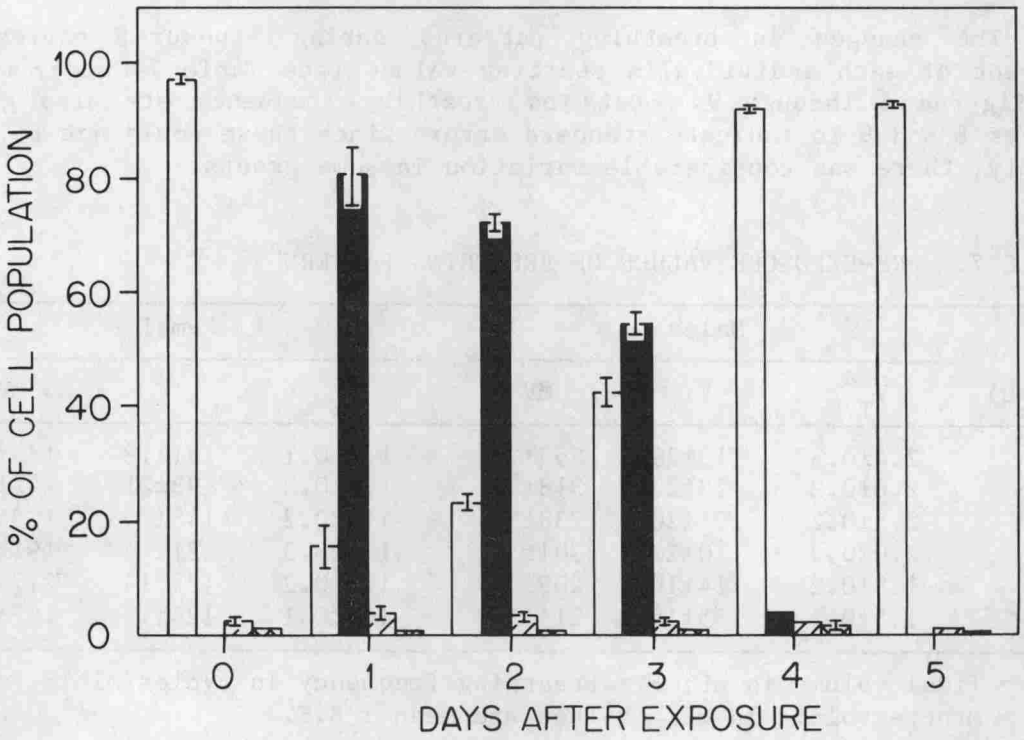

Figure 5

Percent composition of lavaged cell population attached onto coverslips. Cells were identified as alveolar macrophages (□), neutrophils (■), lymphocytes (▧), and others (▨). (Mean ± SE).

There appeared to be a trend in both sexes for total cell number to increase after exposure to at least 2-4 mg/L. The number of lavaged viable macrophages tended to decrease with increasing concentration. There was also a very dramatic change in the percentage of total cells that were alveolar macrophages - approximately 74 percent in the controls versus about 16 percent in those animals treated with 6 mg/L of diesel fuel aerosol for 2 hours. Changes in both alveolar macrophage numbers and total lavaged cells contribute to this shift in ratio.

BREATHING PATTERN DURING EXPOSURE

The changes in breathing patterns during exposure, expressed as percent of each individual's starting values (see Table 7) are summarized in Figures 6 through 9. Data on breathing frequency are also given in Tables 8 and 9 to indicate standard errors since these could not be plotted easily; there was considerable variation in some groups.

TABLE 7. PRE-EXPOSURE VALUES OF BREATHING PATTERN[a]

	Males			Females		
(mg/L)	V_T[a]	F	MV	V_T	F	MV
0	2.4±0.3	113±20	253±28	1.5±0.1	131±18	185±27
0.5	2.6±0.2	123±23	318±78	1.6±0.2	93±21	147±35
1.3	2.5±0.2	101±20	238±35	1.3±0.2	148±25	183±13
2	2.0±0.3	110±27	201±39	1.6±0.3	121± 4	191±18
4	1.8±0.2	114±10	209±36	1.5±0.2	134±14	217±35
6	1.5±0.1	135±15	211±39	1.5±0.1	124±17	187±26

[a]V_T = Tidal volume in mL; F = breathing frequency in cycles/min; MV = minute volume in mL. Values are mean ± S.E.

TABLE 8. BREATHING FREQUENCY IN MALE RATS AT INTERVALS DURING EXPOSURE TO AEROSOLIZED DIESEL FUEL[a]

Concentration (mg/L)	Minutes of Exposure						
	5	10	15	30	60	90	120
0	106± 2	100± 2	100± 6	120±13	104± 3	88±10	109±11
0.5	86±17	93±17	92±18	100±20	100±26	100±19	110±15
1.3	83±12	81±12	73±13	72± 7	68±11	63±10	64±13
2	80± 8	77± 6	76± 6	63± 6	62± 6	53± 9	62± 9
4	83±11	69±11	73±17	63±13	62± 9	53±12	50±11
6	65±16	48± 6	51± 7	45±10	39± 5	36± 9	34± 6

[a]Numbers are percent of individual pre-exposure values, mean ± S.E.

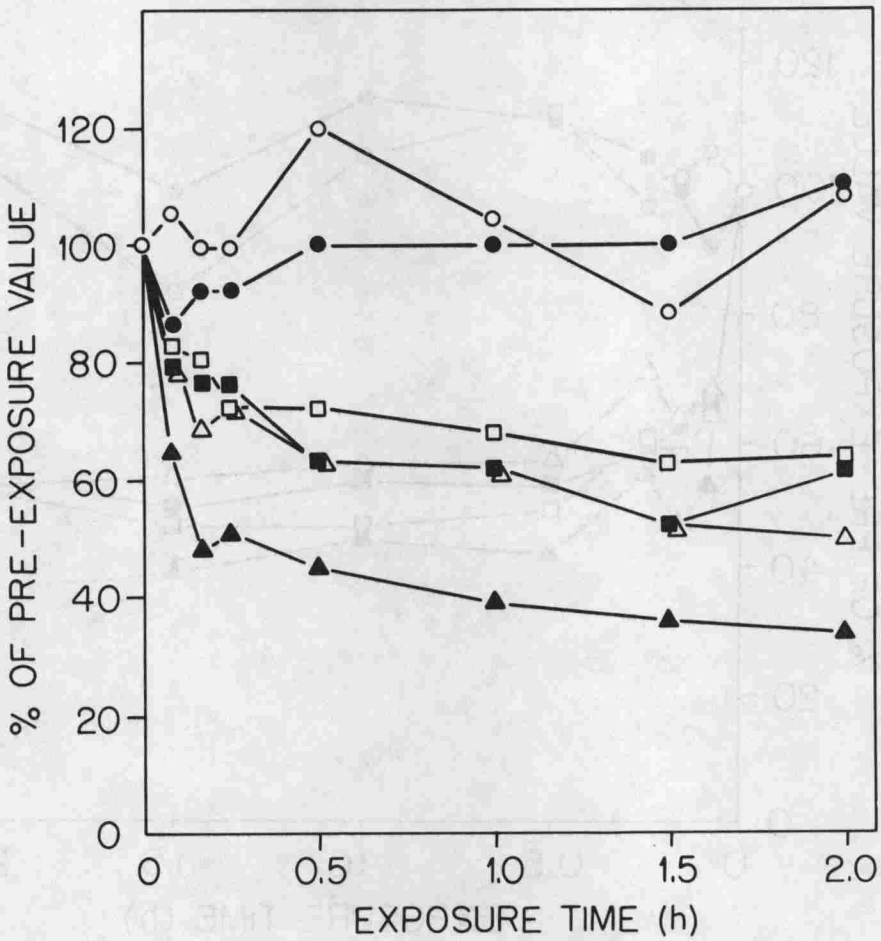

Figure 6

Mean respiratory frequency of male rats during nose-only exposure to diesel fuel aerosol, expressed as percent of individual pre-exposure values. Concentrations (mg/L) were 0 (○), 0.5 (●), 1.3 (□), 2 (■), 4 (△), and 6 (▲).

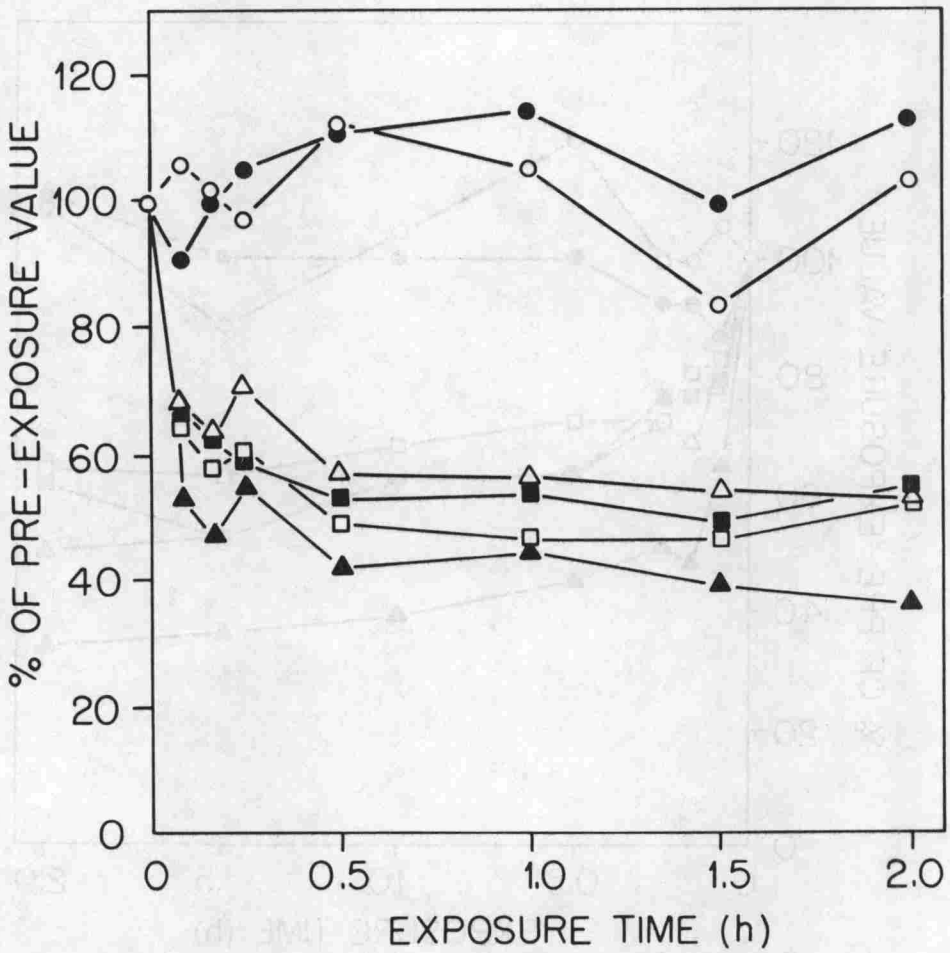

Figure 7

Mean minute volume of male rats during nose-only exposure to diesel fuel aerosol, expressed as percent of individual pre-exposure values. Concentrations (mg/L) were 0 (○), 0.5 (●), 1.3 (□), 2 (■), 4 (△), and 6 (▲).

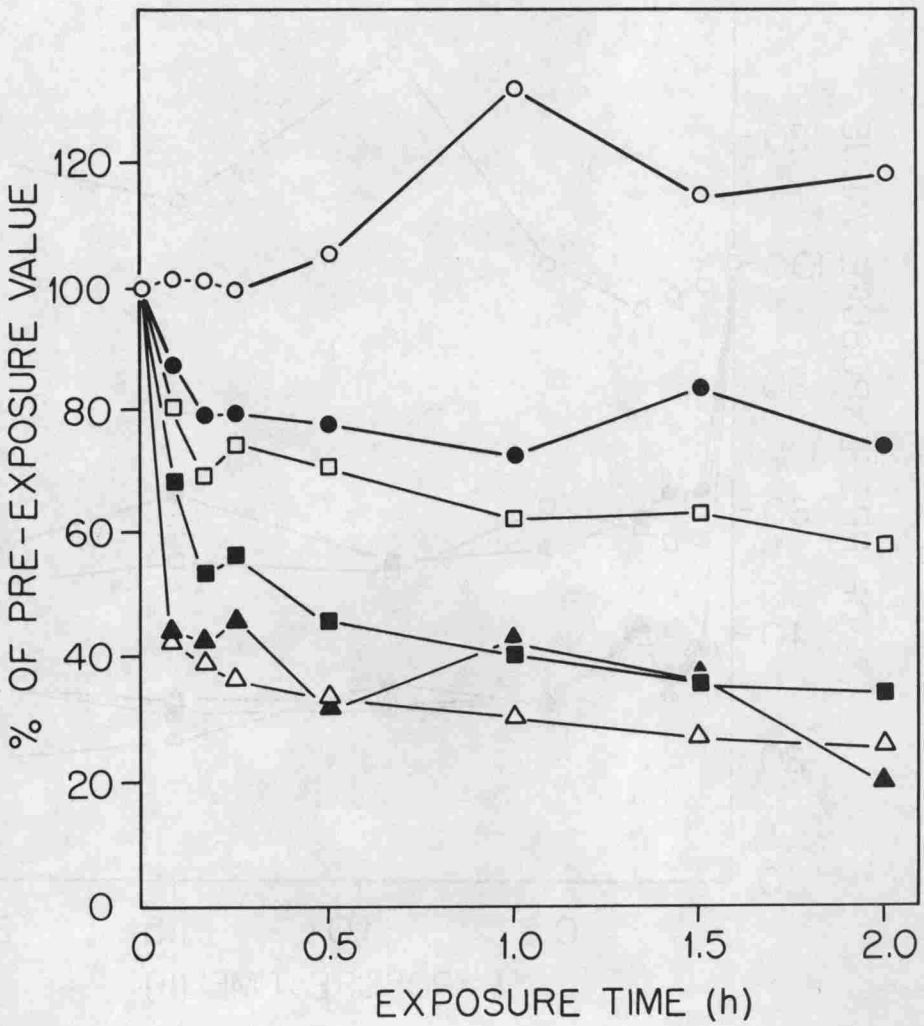

Figure 8

Mean respiratory frequency of female rats during nose-only exposure to diesel fuel aerosol, expressed as percent of individual pre-exposure values. Concentrations (mg/L) were 0 (○), 0.5 (●), 1.3 (□), 2 (■), 4 (△), and 6 (▲).

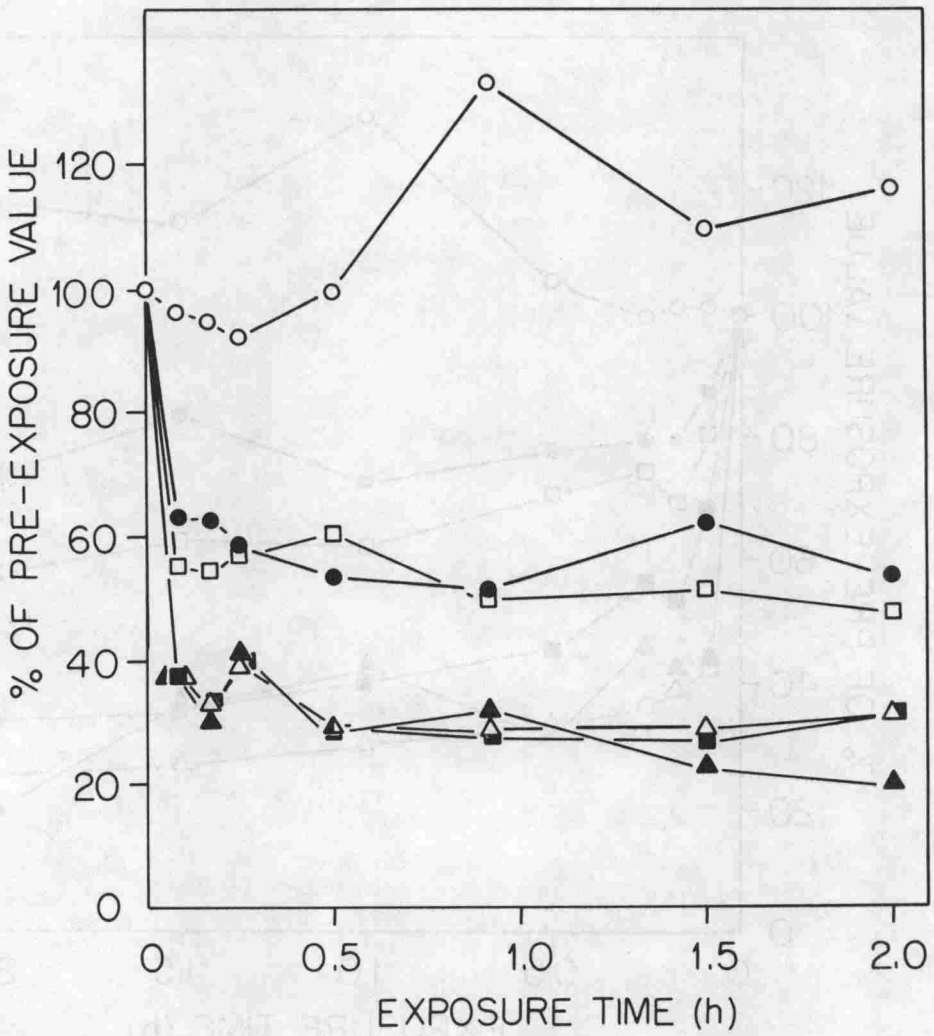

Figure 9

Mean minute volume of female rats during nose-only exposure to diesel fuel aerosol, as percent of individual pre-exposure values. Concentrations (mg/L) were 0 (○), 0.5 (●), 1.3 (□), 2 (■), 4 (△), and 6 (▲).

TABLE 9. BREATHING FREQUENCY IN FEMALE RATS AT INTERVALS DURING EXPOSURE TO AEROSOLIZED DIESEL FUEL (MEAN ± S.E.)

Concentration (mg/L)	Minutes of Exposure						
	5	10	15	30	60	90	120
0	101± 2	101± 4	100± 3	105± 4	132±22	115±15	118±15
0.5	87± 5	80± 7	80±10	78± 8	73± 7	84±18	75±14
1.3	81±12	69±14	75±16	71± 9	63± 9	64±14	59±15
2	68±10	53±13	67± 8	45± 7	40± 6	36± 3	35± 3
4	43± 8	39± 8	37± 8	34± 8	30± 4	27± 1	26± 2
6	44± 4	43± 2	46± 9	32± 7	43±12	37± 9	20± 7

Respiratory frequency has often been used as a prime indicator of sensory irritancy of inhaled substances. As seen in Figure 6, frequency decreased in males in essentially a concentration-related manner. Exposure to 0.5 mg/L resulted in little effect, if any, while frequency was reduced to about 40 percent of pre-exposure values by inhalation of 6 mg/L. There was some compensation in that tidal volume tended to decrease among rats exposed to lower concentrations and to appear more normal among rats exposed to higher concentrations. Thus minute volume, the product of frequency and tidal volume, showed less relation to aerosol concentration than respiratory frequency did (Figure 7). The overall reaction of the females was quite similar (Figures 8 and 9, Table 7), although they appeared to be slightly more sensitive than the males. Both respiratory frequency and minute volume decrease rapidly during the first 30 minutes of exposure and then show only very moderate decreases, if at all, during the remaining 90 minutes of exposure. This appears to hold regardless of whether the animals are exposed by the nose only method (Figures 6 to 9) or by the whole body method (Figures 10 and 11). As seen in Table 10, breathing frequency (data at 30 min of exposure) decreased in essentially a concentration-related manner in both sexes, despite considerable variation within each group.

TABLE 10. MEAN RESPIRATORY FREQUENCY AFTER 30 MINUTES OF EXPOSURE (NOSE-ONLY) TO AEROSOLIZED DIESEL FUEL, EXPRESSED AS PERCENT OF PRE-EXPOSURE VALUE (MEAN ± S.E.)

mg/L	Male	Female
0	107 ± 3	105 ± 4
0.5	100 ± 20	78 ± 8
1.3	72 ± 7	71 ± 9
2	63 ± 6	46 ± 7
4	63 ± 12	34 ± 8
6	45 ± 10	32 ± 6

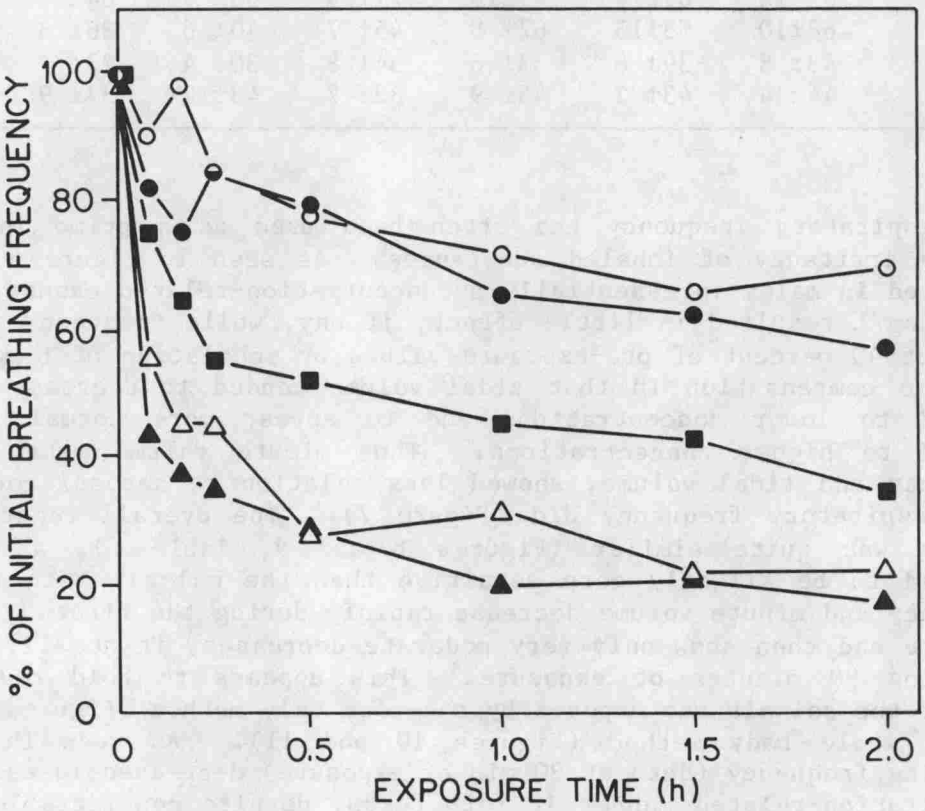

Figure 10

Mean respiratory frequency of female rats during whole-body exposure to diesel fuel aerosol, expressed as percent of individual pre-exposure values. Concentrations (mg/L) were 0 (○), 0.5 (●), 2 (■), 4 (△), and 6 (▲).

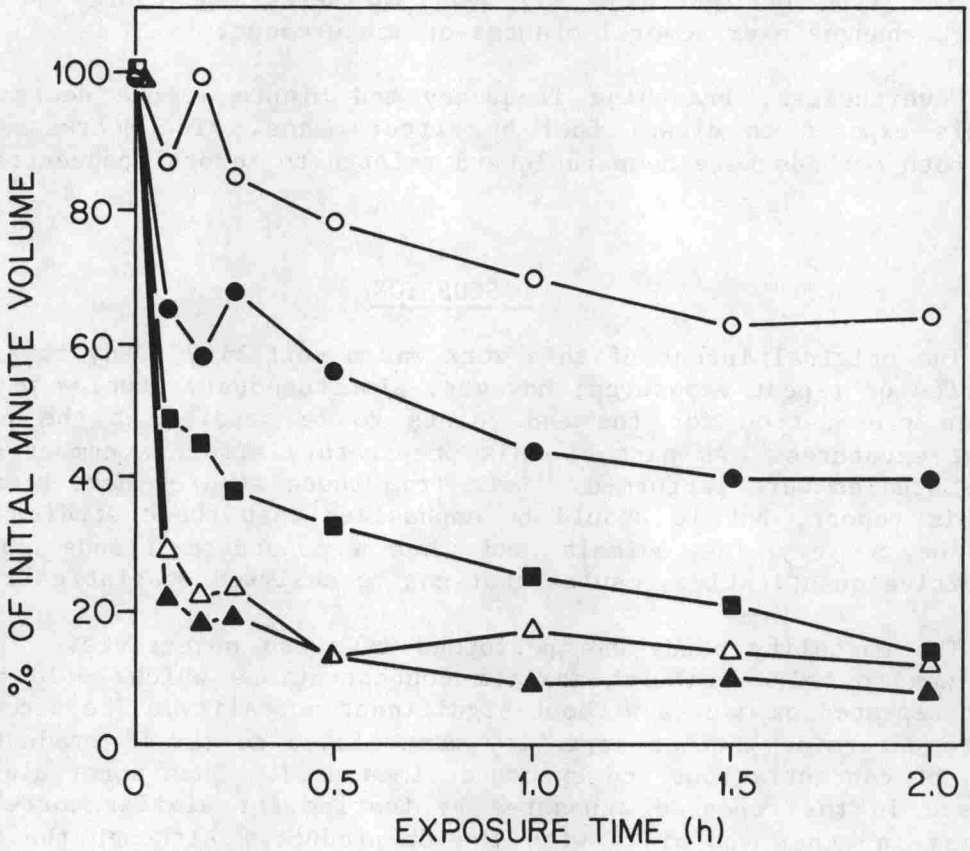

Figure 11

Mean relative minute volume of female rats during whole-body exposure to diesel fuel aerosol, expressed as percent of individual pre-exposure values. Concentrations (mg/L) were 0 (○), 0.5 (●), 2 (■), 4 (△), and 6 (▲).

Data from the whole-body exposures were qualitatively similar except that breathing frequency and relative minute volume decreased in the unrestrained control animals, as shown in Figures 10 and 11, respectively. Similar data from two separate control groups of 8 animals were pooled for these figures. The decreases observed in unrestrained control rats and the slight increases observed in the nose-only exposures may be a result of reaction to restraint during the measurement of breathing pattern. We have previously observed less dramatic decreases in minute volume over an hour with F-344 rats restrained by a cuff around the neck (unpublished data). Thus the type of restraint may well influence the course of breathing pattern changes over several minutes of measurement.

Nevertheless, breathing frequency and minute volume decreased among animals exposed to diesel fuel by either means. The decreases observed with both methods were comparable and related to aerosol concentration.

DISCUSSION

The original intent of this work was a mortality study to prepare for a series of repeat exposures; however, simultaneously studies were carried out in preparation for the end points to be studied at the end of the repeat exposures. As part of this preparatory effort a number of limited acute studies were performed. Data from these studies have been included in this report, but it should be emphasized that these studies were only done on a very few animals and thus demonstrate trends rather than definitive quantitative results that can be analysed statistically.

The mortality study was performed with two objectives. The primary goal was to help establish maximum concentrations which could be used in later repeated exposures without significant mortality. The secondary goal was to determine whether mortality was related to the Ct product over the range of concentrations and exposure times used. Such a correlation could be used in the repeated exposures by testing for similar correlations of changes in other endpoints with the Ct product. Although the Ct product can be a useful index of exposure (1, 8), it must be used with caution (9).

Mortality was found to be highly related to the Ct product, and a lower confidence bound of 8 mg.h/L was estimated for 1 percent mortality after single exposures. This value was at two standard errors below an estimate of the Ct product expected to result in 1 percent deaths. Thus 8 mg.h/L could be considered an approximation of a "maximum tolerated dose". The particulate concentrations for this Ct product would be 4 mg/L for a 2 hour exposure and 1.3 mg/L for a 6 hour exposure.

Since the primary interest of this study was to develop a response surface over a range of concentrations and durations of exposure, no attempt has been made to define LC50's for different exposure times. Similarily, since it was not the intent of this study to compare diesel fuel from different sources, no extensive commentary on the early experiments will be made. The mortality data obtained from the commercial fuel

was used to assist in planning of the more extensive mortality study with the reference grade Phillips fuel.

The two ancillary studies reported here dealt with effects of acute exposure on the respiratory tract. In the first study, a transient increase in pulmonary free cell number (influx of granulocytes) was observed, with a return to control values by one week after exposure. Such an influx of cells is not unique to diesel fuel aerosol, but has been observed with other particles (10). An exposure of greater than 2 mg/L and probably less than 4 mg/L for 2 hours appears to be required to produce a readily demonstrable increase in pulmomary free cell number, indicating that high concentrations were required to produce these large influxes of cells into the lung.

The intent of the experiments on breathing pattern was to demonstrate whether the diesel fuel aerosol was a respiratory irritant which could influence the breathing pattern over the range of concentrations used in our other exposures and potentially with human exposure, and furthermore, whether breathing frequency and minute volume changed in a concentration-related manner over this same range of concentrations.

Changes in breathing pattern during exposure were more readily observed at lower concentrations. Breathing frequency and minute volume often decrease in response to the sensory irritancy of gases and aerosols (5), perhaps as a defense mechanism to help decrease inhalation of noxious materials (6). Thus this response once again is not specific to diesel fuel but represents a more generalized reaction to irritants. The concentration of airborne materials which results in a 50 percent depression of breathing frequency in mice after 10 minutes of exposure (RD50) has been proposed by Alarie and others to represent a severely debilitating concentration in man (11). Levels resulting in a 5 percent reduction in respiratory rate in mice would produce slight irritation in man. RD50 values for a few common irritants are 1.7 ppm for acrolein (11), 3.1 ppm for formaldehyde (11), 9.3 ppm for chlorine (12), 130 ppm for sulfur dioxide (13), and 309 ppm for hydrogen chloride (12).

Many of the irritancy studies have been performed with mice, although rats have also been used (6). In this particular case, the RD50 for naive F-344 rats exposed to formaldehyde was 31.7 ppm and 4.9 ppm for B6C3F1 mice, representing an obvious species difference. Our data are exclusively from the Sprague-Dawley rat. Since the relationship of breathing frequency at 10 minutes to aerosol concentration did not appear to be influenced by the method of exposure, data from nose-only and whole-body exposures were pooled.

Results are shown in Figure 12. There was a linear relation between aerosol concentration and depression of respiratory frequency. The RD50 value was 3.75 mg/L. This value is obviously far greater than many of those previously cited for respiratory irritants. It has been proposed (11,14) that 0.1 RD50 be used as the ceiling value permitted in industrial exposures. This value for diesel fuel aerosols, 0.4 mg/L, is within values expected for human exposures.

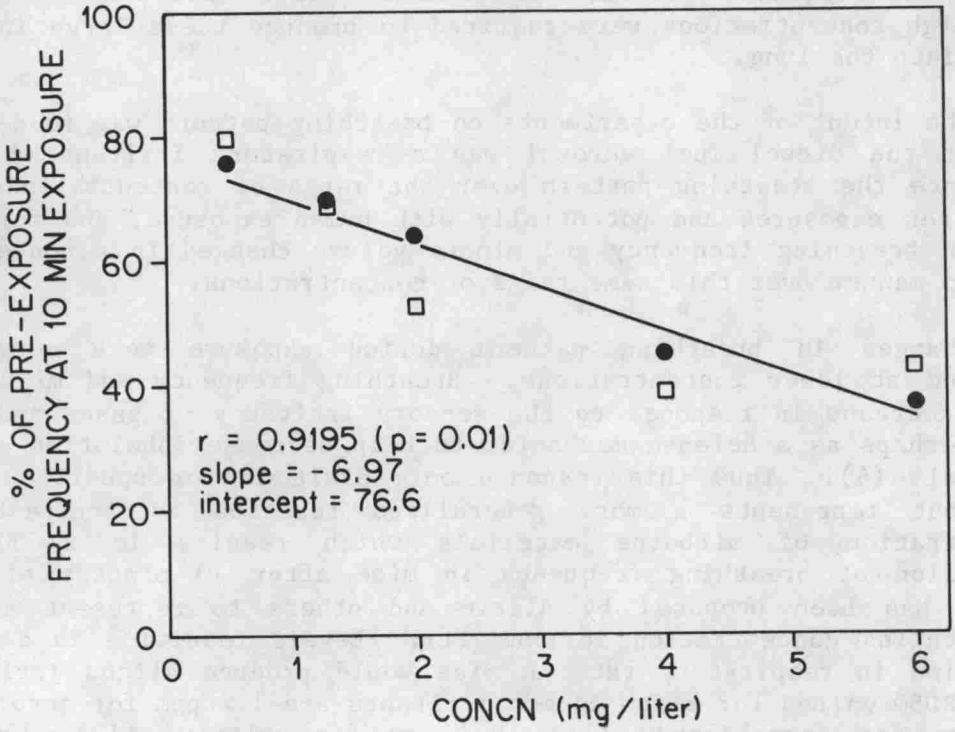

Figure 12

Linear dose-response curve for breathing frequency at 10 minutes of exposure to various concentrations of diesel fuel particles. All animals were females. Exposures were either nose-only (□) or whole-body (●).

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APPENDIX

TABLE 1A. MORTALITY BY SEX AMONG GROUPS OF 10 RATS AFTER SINGLE EXPOSURES IN FIRST TWO BLOCKS OF EXPOSURES TO COMMERCIAL FUEL AEROSOL

Concentration (mg/L)	Hours of Exposure			
	1	2	4	6
1.33				1F 1M,1F
2			3M 1M,2F	3M,2F
2.67				5M,5F
3			5M,3F 4M,3F	
4		1M,2F	5M,4F	
6		1F 1M,5F		
8		1M		
12	1M			
16	1M,1F			

TABLE 2A. MORTALITY AMONG GROUPS OF 10 RATS AFTER SINGLE EXPOSURES IN THE FIRST TWO BLOCKS OF EXPOSURES TO COMMERCIAL FUEL AEROSOL EXPRESSED IN TERMS OF CT PRODUCT

Ct (mg.h/L)	Mortality[a]
4	0
8	1, 2, 3, 3, 3
12	5, 8, 9, 1, 6, 1
16	10, 9, 1, 2

[a]Each number represents the number of rats that died in an exposure group of 10 animals.

TABLE 3A. MORTALITY BY SEX AMONG GROUPS OF 10 RATS AFTER SINGLE EXPOSURES IN LAST BLOCK OF EXPOSURES TO COMMERCIAL FUEL AEROSOL

Concentration (mg/L)	Hours of Exposure			
	1	2	4	6
0.67				0
1			0	
1.33				
2		0,0		
2.67				0
				1F
				1M,1F
				1M,5F
3				
4				0
6				0
8		0		
12				
16				

TABLE 4A. MORTALITY BY SEX AMONG GROUPS OF 10 RATS AFTER SINGLE EXPOSURES TO AEROSOLIZED PHILLIPS DIESEL FUEL.

Concentration (mg/L)	Hours of Exposure		
	2	4	6
2.67			0
4		0	2M,1F
5.33			1M,2F
6		0	
		0	
8	0	2M,2F	2M,4F
	1M		
12	2F	2M,4F	5M,5F
14	1M,4F		
16	5F	2M,4F	5M,5F

TABLE 5A. MILLIONS OF LAVAGED PULMONARY FREE CELLS AT 48 HOURS AFTER A 2 HOUR EXPOSURE TO VARYING CONCENTRATIONS OF AEROSOLIZED DIESEL FUEL (MEAN ± S.E.) SHOWING THE DATA BY SEX

			Total Cells	Alveolar Macrophages
mg/L	Sex	Body weight (g)	Kg body weight	Kg body weight
0	M	461 ± 17	8.6 ± 0.4	7.5 ± 0.5
	F	253 ± 5	16.0 ± 3.3	11.3 ± 1.7
1.33	M	484 ± 20	19.4 ± 1.3	12.0 ± 2.0
	F	251 ± 9	13.5 ± 1.8	11.0 ± 1.3
2	M	461 ± 32	12.8 ± 2.3	9.2 ± 2.8
	F	255 ± 8	20.8 ± 5.3	7.2 ± 1.0
4	M	508 ± 16	17.4 ± 1.5	4.4 ± 2.6
	F	256 ± 12	35.3 ± 11.8	5.1 ± 1.3
6	M	453 ± 56	16.9 ± 1.8	2.4 ± 0.1
	F	245 ± 6	32.6 ± 3.2	5.3 ± 0.8

PERSONNEL

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Principal Investigator	:	Walden Dalbey, PhD.
Research Scientist	:	Simon Lock, PhD.
Technicians	:	Susan Garfinkel Timothy Ross Edna Stout
Statisticians	:	Richard Schmoyer, PhD. Alan Zinsmeister, PhD.

The following personnel from the Analytical Chemistry Division were responsible for aerosol generation and monitoring.

Michael Guerin, PhD.
Robert Holmberg, PhD.
Roger Jenkins, PhD.
Jack Moneyhun

PUBLICATIONS

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