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The research carried out under this contract was concerned with direct interaction between living mammalian cells and airborne pollutants. Mammalian cell lines were grown on cellulose ester membranes (Millipore) and exposed to test atmospheres in a specially designed system which maintained the cells in a viable state and in which pollutant concentrations were controlled and monitored.

Specific tasks for this year have been to:

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20. ABSTRACT (continued)

a. Modify the exposure system to permit the use of organic vapors such as hydrazine and jet fuels.

b. Measure the cytotoxic effects of mixtures of NO_2 and O_3 as well as hydrazine and jet fuels on human and animal cell lines. Effects on cell proliferation and DNA synthesis would be included.

c. Initiate studies to test the hypothesis that atmospheric oxidant gases such as NO_2 or O_3 may react with hydrocarbon vapors, such as jet fuels, to produce toxic or mutagenic products.

d. Evaluate two cell strains from adult rat lung tissues for use in the exposure system studies.

The use of organic vapors in the exposure system has been facilitated by the employment of resistant materials such as stainless steel, Teflon, and silicone rubber as appropriate. The modifications have allowed generation of atmospheres containing aircraft fuels, hydrazine, and ethylmethane sulfonate. At the present state of development, the system can be arranged for small-scale exposures to single organic compounds with the addition of either O_3 or NO_2.

Measurements of the effects of NO_2 and O_3 on cell cultures which were initiated in previous years have been continued. Cytotoxicity has been the most obvious effect of the gases. Other effects such as inhibition of DNA replication or chromosomal damage have not been significant. This may be because cytotoxicity is marked at very low concentrations of the gases, i.e., at 0.03 ppm of O_3 or 0.15 ppm of NO_2, the toxic effects overshadow any possible genetic damage. At lower concentrations, accurate generation and measurement of gases are also problems. Because no genetic effects have been observed with either gas under the present exposure conditions, studies of gas mixtures have not been undertaken. Evidence from other sources which became available during the year indicated that hydrazine was not mutagenic in cell cultures and toxic only at high concentrations. Therefore, extensive studies with this material did not seem useful.

Aircraft fuels (e.g., JP-5) were moderately toxic to cells in culture. No increase in the toxicity was noted when the JP-5 was exposed to O_3 or before exposing cells in culture to 1 mg/ml for one hour.

Two cell strains previously isolated from adult male Sprague-Dawley rat lungs have been characterized for possible use in the exposure system. These cells retain certain characteristics of normal alveolar epithelial lung cells, and as such may enable study of some lung cell functions in vitro. The cells can be maintained under the conditions necessary for exposure to gaseous materials, and some studies have been done on the effects of O_3 and NO_2 on these cells. Additional studies have included growth factor requirements and benzo(a)pyrene metabolic studies. The cells are very sensitive to low concentrations of O_3 and NO_2, as found for other cell lines. They require enriched medium containing hydrocortisone for optimal growth. The cells can convert benzo(a)pyrene, a known lung carcinogen, to hydroxylated derivatives.

DIRECT EXPOSURE OF MONOLAYERS OF MAMMALIAN CELLS TO
AIRBORNE POLLUTANTS IN A UNIQUE CULTURE SYSTEM

SUMMARY

FINAL

The research carried out under this contract was concerned with direct interaction between living mammalian cells and airborne pollutants. Mammalian cell lines were grown on cellulose ester membranes (Millipore) and exposed to test atmospheres in a specially designed system which maintained the cells in a viable state and in which pollutant concentrations were controlled and monitored.

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PREFACE

This is the third and final annual report submitted under AFOSR Contract No. F49620-79-C-0083. Studies of the effects of O_3 and NO_2 on mammalian cells are presented, and the present capabilities of the cell culture exposure system are described.

This report summarizes the work accomplished during the two grant years (AFOSR-77-3343) prior to the final contract year, and presents in detail the experimental studies in the final year.

This project was carried out in the Department of Community and Environmental Medicine, University of California, Irvine. The Principal investigator was Ronald E. Rasmussen, Ph.D., Associate Adjunct Professor; Co-principal investigator was T. Timothy Crocker, M.D., Professor and Chairman, Community and Environmental Medicine. Project monitor was William O. Berry, Ph.D., of the Life Sciences, Directorate, AFOSR.

SECTION I. Exposure System Modification

Introduction

The cell exposure system used in these studies was originally designed to be used with the oxidant gases NO_2 and O_3, and possibly with SO_2. The materials used in construction were therefore selected to be compatible with these gases. Exposure chambers were fabricated from Lexan plastic; gas lines and other plumbing were of stainless steel or teflon; the special cell culture holders were made of Lexan plastic. The details of these exposure chambers have been described in previous reports.

Physically, the exposure system is very sturdy. As development of the system has proceeded, it has taken on a modular character. Lexan plastic and stainless steel chambers and cell culture holders are readily interchangeable. Additional chambers have been developed and used for exposure of organ cultures or rodent tracheal explants. The gas flow capacity of the system is sufficient for acute exposure studies with small numbers of mice or rats. This may enable direct comparison of in vivo and in vitro effects in the same system.

During contract year 1978-1979, exposure chambers were assembled and lined with stainless steel foil, and cell culture holders were machined from stainless steel rod in preparation for studies with organic solvents. These chambers have also proven useful in studies with O_3 and NO_2.

In the present year, the stainless steel chambers and culture holders have been used to study the effect of fuel vapors (JP-5) on cell cultures. Methods have been developed for vaporizing JP-5 or other similar hydrocarbons, mixing them with air, ozone, or other gas and either condensing the fuel vapors or allowing the vapors to contact the cell cultures.

The studies described below have demonstrated the flexibility of the system, in that, with minor modifications, a variety of potential airborne pollutants can be vaporized and their toxic effects on cell cultures measured.

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Experimental Studies

Summary Description of Cell Exposure System.

The system was designed to resemble to some degree the surface of the mammalian respiratory epithelium. This is accomplished by planting cells on cellulose membrane filters (Millipore) and assembling the filter into a special holder (Figure 1) which allows perfusion of growth medium through the filter from the side opposite the cells so that they are nourished and kept moist. Growth medium perfusing through the filter is drawn off by a peristaltic pump so that a layer of medium does not build up over the cells. Figures 2 and 3 show stainless steel filter holders, but similar holders have been made from Lexan. The latter are useful for hydrazine exposures where metal is not acceptable (1).

Gas exposure chambers have been constructed of Lexan plastic (Figure 4) and wood lined with stainless steel foil (Figure 5). These chambers provide exposure capability for oxidant gases as well as organic solvents and fuels, and hydrazines. A typical arrangement for exposure of cells to a mutagenic agent is shown in Figure 6. The vapors leaving the chamber are completely trapped by a series of gas washing bottles and no materials are released to the environment. For exposure to the oxidant gases O_3 and NO_2, a more elaborate system is used (Figure 7). The concentrations of the latter gases are in the same range as found in ambient polluted air (O_3 0.03-1 ppm; NO_2 0.15 ppm) and therefore no significant laboratory or environmental hazards are present. The effluent from the exposure chambers is vented through a chemical fume hood.

Summary of Previous Studies.

Cytotoxic effects of NO_2 or O_3 were measured by planting dispersed cells (Line V79, Chinese hamster lung fibroblasts) on the membrane filters and exposing to the test gas. The toxic effect was measured by incubating the filters for 6 to 8 days following exposure, and counting the number of macroscopic colonies of cells developed on the
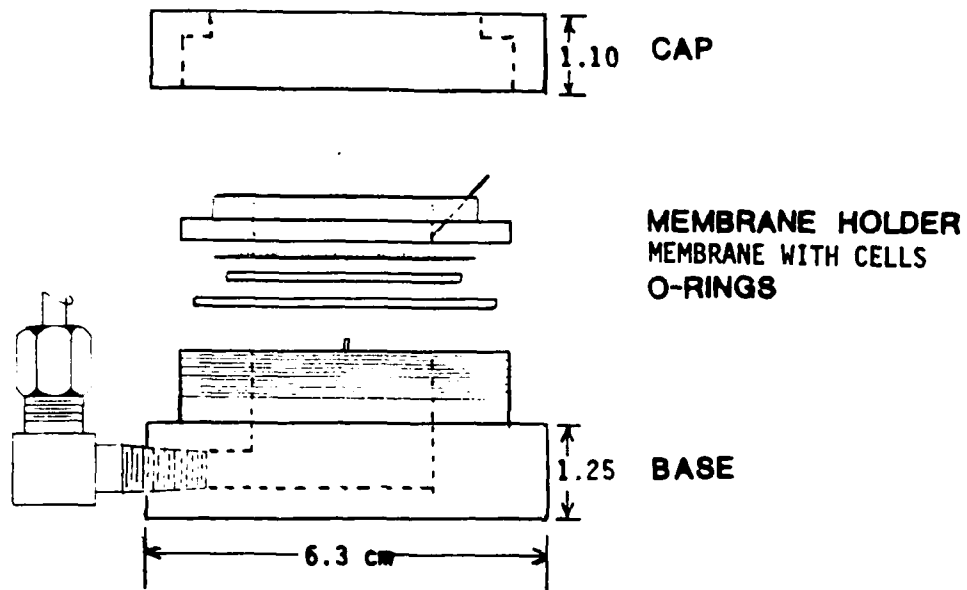
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FIGURE 1. Diagram of filter holder for holding Millipore membrane filters during exposure to gases or vapors. Holders have been fabricated from Lexan plastic and stainless steel.
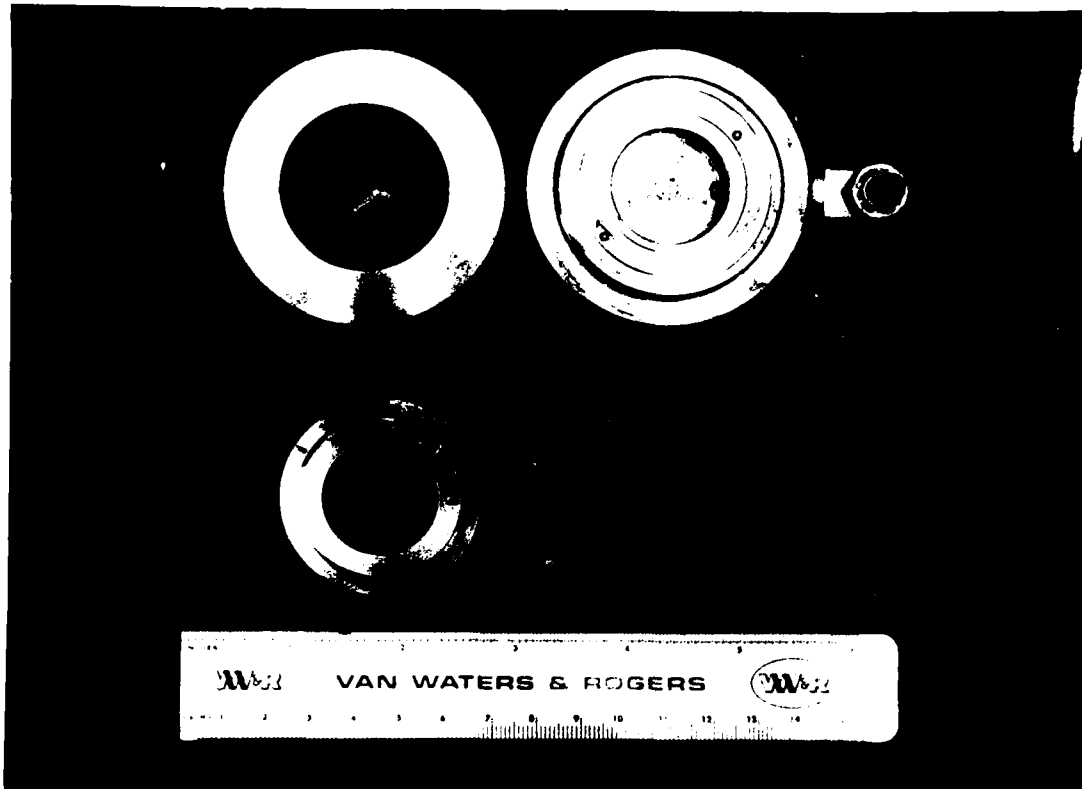
FIGURE 2. Filter holder of stainless steel disassembled to show components.
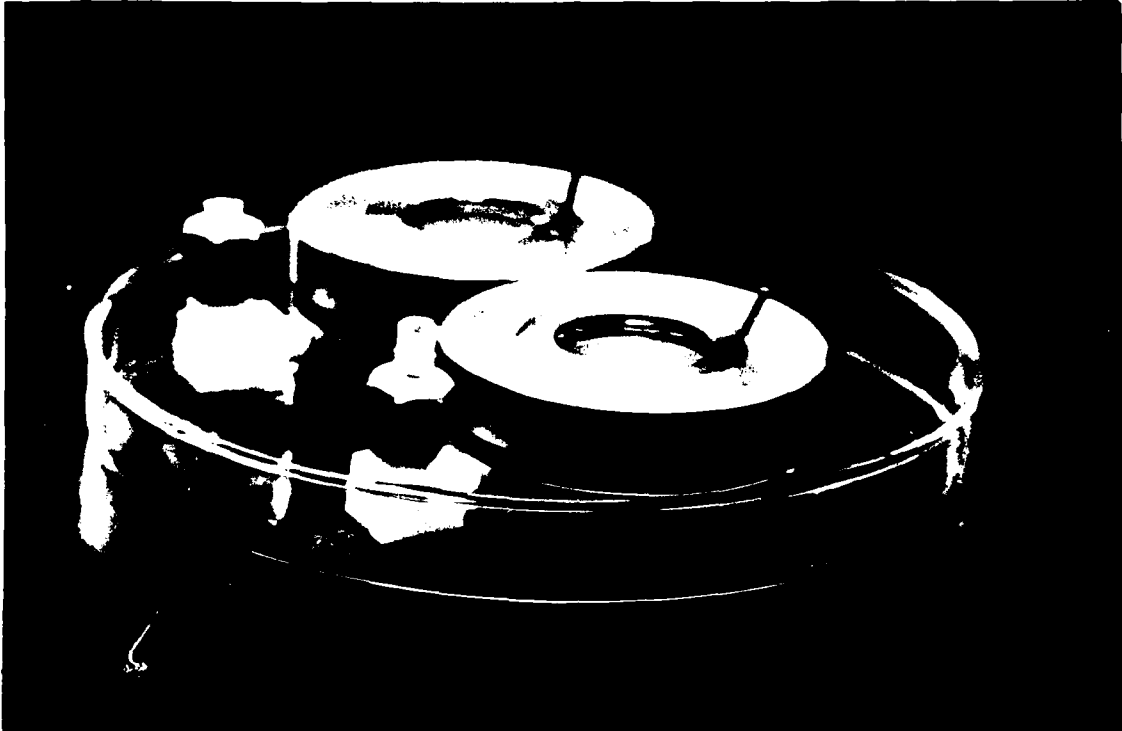
FIGURE 3. Stainless steel filter holders assembled with filters in place ready for placement in the gas exposure chamber.
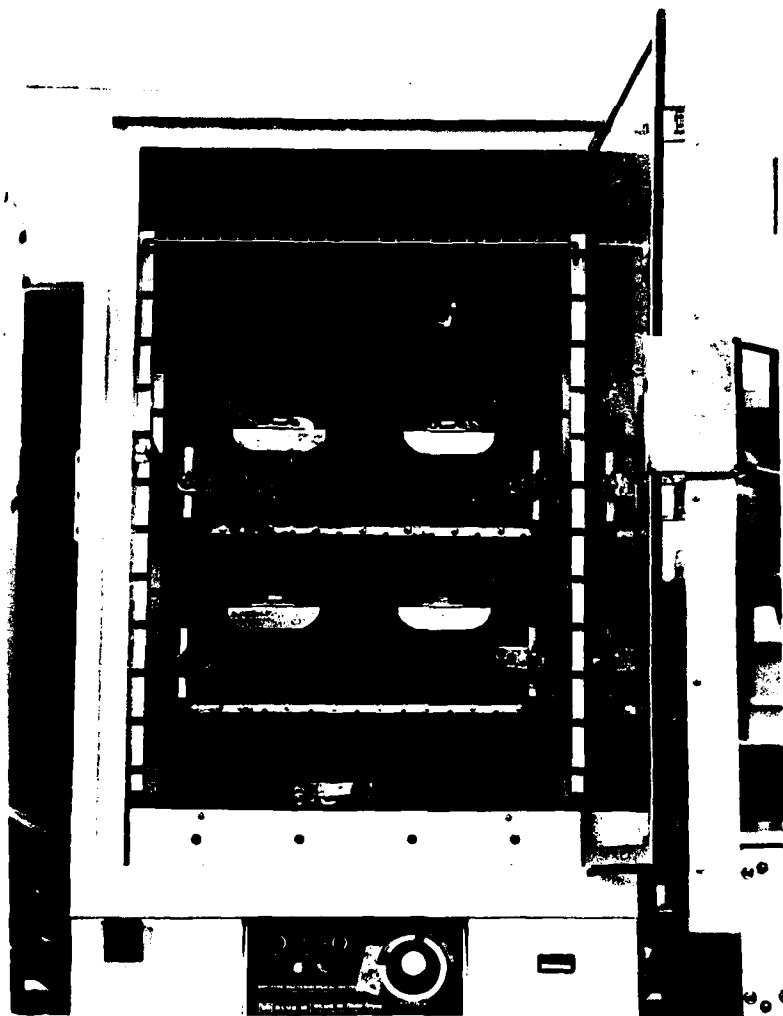
FIGURE 4. Lexan cell exposure chambers enclosed in 37° incubator for use with NO_2 and Ozone. Ozone generator is at the right rear of the incubator chamber. Carrier gas (5% CO_2:95% air) mixed with NO_2 enters through the coil of stainless steel tubing at the top of the chamber. Culture medium enters via tubing through a port in the righthand wall.

FIGURE 5. Stainless steel cell filter holders in place inside a stainless steel lined gas exposure chamber. Nutrient cell growth medium enters through tubes at the top of the chamber. Medium which perfuses through the filters is drawn off through the tubes at the rear wall of the chamber. The test gas enters at the left end of the chamber and exits at the right end.

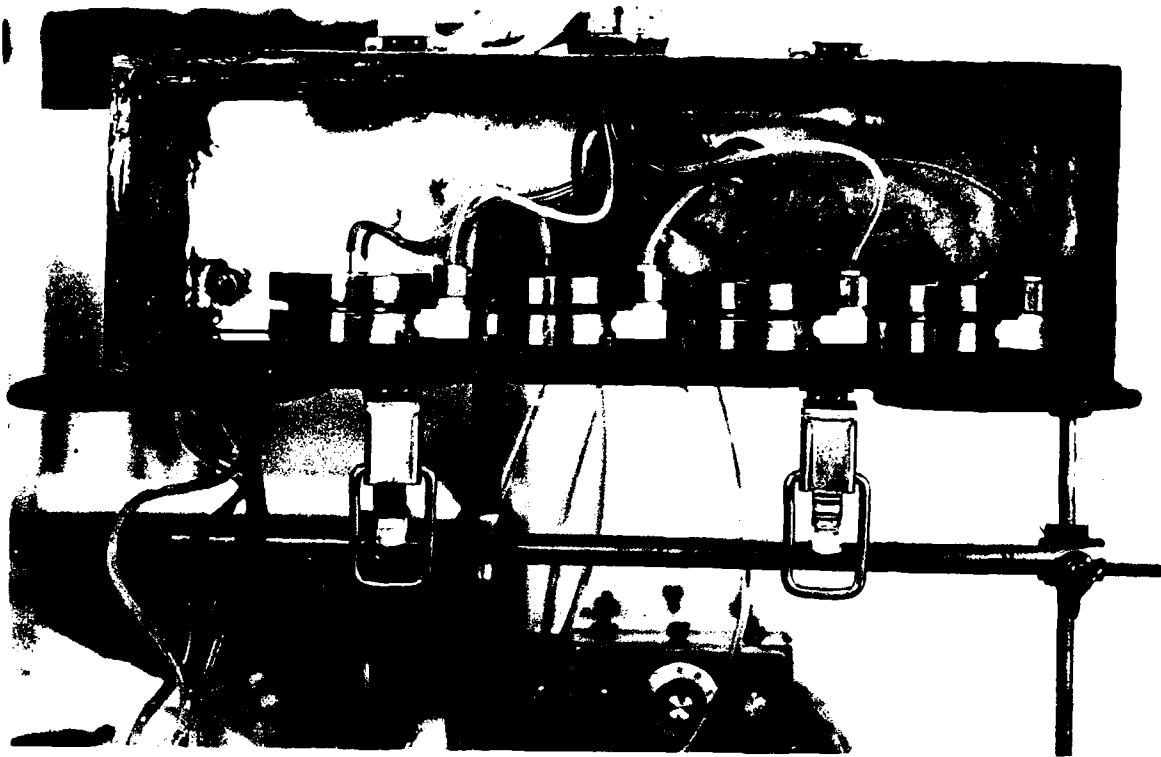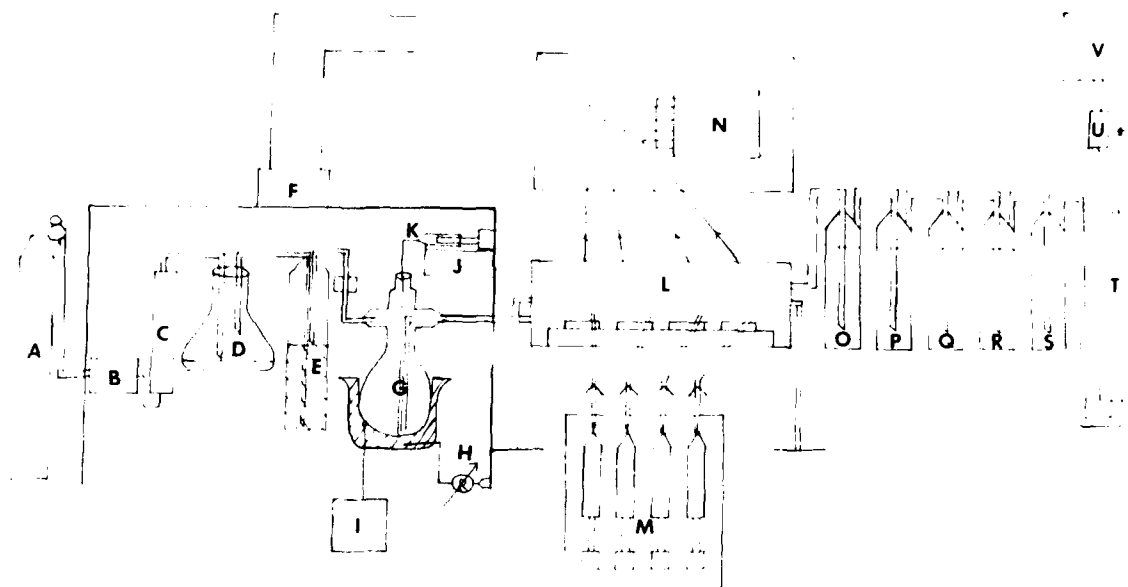FIGURE 6. Generation and exposure system for atmospheres containing mutagenic chemicals. The system shown is in use for producing atmospheres containing ethylmethane sulfonate. A, carrier gas cylinder; B. glass wool filter; C, rotameter; D, flask with water to produce moderately humid atmosphere for activation of Purafil; E, Purafil column; F, vent fan for fume hood; G, vaporization and mixing chamber; H, rheostat for heating mantle; I, thermometer; J, syringe pump; K, capillary tube for introducing mutagenic chemicals into the mixing chamber; L, cell exposure chamber; M, syringe pump for cell growth medium; N, peristaltic pump for removal of cell growth medium from cell surface; O-S, gas washing bottles for trapping mutagenic vapors; T, rotameter; U, coarse adjustment for vacuum; V, vacuum pump.

FIGURE 7. Six-chamber system for exposing cell cultures to NO_2, O_3, or SO_2.

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filters. Controls included cells exposed to clean air and cells on filters never exposed to gas or air. Previously reported studies (2,3) showed that both NO_2 and O_3 were highly toxic to these cells. An exposure of 6 hr to 0.15 ppm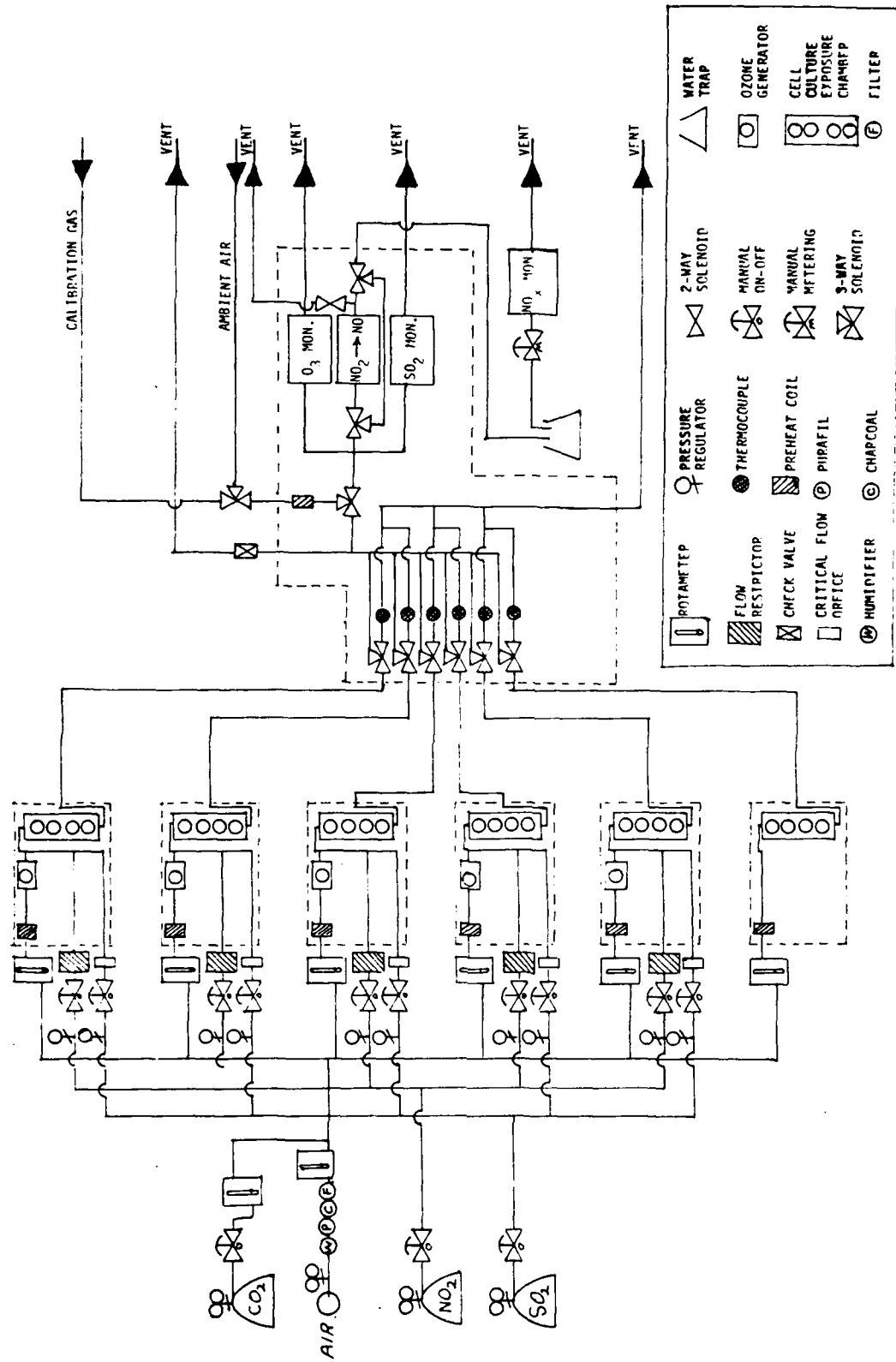 of NO_2 or 0.05 ppm of O_3 caused a loss of over 90% of the colony-forming ability. Studies of the mechanism of action of NO_2 and O_3 indicated that cells were either disintegrating as the result of the exposure or becoming detached from the filters and thus being lost (3).

Preliminary tests for genetic effects of these gases were either negative or inconclusive. The high sensitivity to the toxic effects of the gases limited the exposure, and hence the total dose that could be delivered to the cells. More extensive tests are described in the present report.

Methods for Fuel Vapor Generation and Containment.

The system developed for volatilization of aircraft fuel (JP-5) was patterned on that used at the Toxic Hazards Research Unit (THRU) at WPAFB, Ohio. Liquid fuel was injected, using a syringe pump, into the top of a heated column (25 x 1.3 cm) of 6 mm glass beads through which a current of air was continuously passed from bottom to top. The volatilized fuel was then carried through an exposure chamber or through a cold trap in which possible oxidation products of the fuel were collected. The temperature of the glass bead column was maintained between 95° and 100° using heat tape and a rheostat. Gas flow through the column was dependent on the specific experiment underway, and ranged between 500 ml to 2 l per minute. The carrier gas also depended on the experiment. For exposure of cell cultures to fuel vapors, the carrier gas was room air which had been passed through a series of filters to remove bacterial and chemical pollutants prior to entering the system. The filter train consisted of a packed cotton filter, a Purafil (4) column, an activated charcoal column, and a Millipore 0.45 um bacteriological filter. The apparatus was enclosed within an acrylic hood which was vented through a conventional chemical fume hood. The total amount of fuel involved at any one time was in the range of 5 - 10 ml.

Ozonized fuel was prepared by passing clean air through an ultraviolet ozonizer (Hamamatsu Model C940) prior to passing through the heated vaporization column. The maximum ozone concentration attainable was one ppm at a flow rate of 2.0 liters/min. The effluent from the column was collected in a cold-finger trap immersed in a solid CO_2-acetone bath.

Although tests showed that Lexan plastic was resistant to the solvent effects of fuels, it did tend to absorb and slowly release the fuel. Therefore all cell culture exposures to fuel vapors were done using stainless steel chambers and cell culture holders.

System Evaluation

Fuel volatilization.

Because aircraft fuels are made up of hydrocarbons with a range of vapor pressures and boiling points, the vapor generated in the system did not have the same composition as the liquid fuel. The glass-bead column acted as a fractionation device, and high-boiling materials tended to condense on the beads and not be carried into the exposure chamber. This fractionation has also been observed at the THRU in connection with vaporization of fuels for animal exposures (5). The cell culture studies (described below) included both the "volatile" and "non-volatile" fractions.

For single chemical species, this problem was not encountered. The injection of materials into the vaporization column or flask as previously described could be controlled so as to assure complete volatilization.

Mammalian Cell Exposure Application.

The exposure system for organic vapors is limited to a single exposure chamber. Although technically it would be possible to expand this capacity, it does not appear appropriate to do so for a number of reasons; the major one being that each test material

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is likely to require special modifications of the system. Secondly, as will be evident from the cell exposure studies described below, the mode of cell exposure may exaggerate the sensitivity of cells well beyond that which is present in vivo. This may indicate an important application of the exposure system in studies of pollutant-cell membrane interactions on a morphological level, using electron microscopy. However, studies of mutagenesis, or other events requiring cellular metabolism may be overshadowed by the toxic effects resulting from the impact of airborne pollutants on the cell surface. This tentative conclusion is based on studies with established mammalian cell lines, and may not apply to primary cultures of cells which retain their in vivo characteristics.

In summary, the exposure system is now at a stage of development that allows generation and monitoring of a variety of materials, mixing them with ozone or NO_2, and exposure of cell cultures to the resulting vapor or condensed material. The system is probably best suited for laboratory research studies rather than as a device for screening for possible biological effects of test materials. Such research studies are continuing with separate support.

SECTION II. Cell Culture Studies

Introduction

The goals of the cell culture studies for the present year have been:

To measure cytotoxic effects and possible effect on DNA synthesis of NO_2, O_3, and their mixtures, as well as the effect of selected fuels;

To test whether oxidant gases may interact chemically with hydrocarbon fuels to form toxic compounds;

To incorporate additional rat lung cell lines into these studies.

The studies of the cytotoxic effects of oxidant gases represent a continuation of previous work. They have been extended to include effects of O_3 or DNA synthesis and sister chromatid exchange initiation. Studies of mixtures of NO_2 and O_3 have not been undertaken, the reason being that very low levels of NO_2 or O_3 are so cytotoxic that the generation and monitoring of mixtures of the two gases at even lower concentrations would present technical problems not readily solved with the present equipment. In any case, it is debatable whether the effects produced by oxidant gases on cells in vitro are indicative of in vivo hazards.

Because fuel vapors and oxidant gases may both be present in occupational settings, the formation of novel toxic compounds may be possible. This hypothesis was tested by mixing fuel vapors and O_3 or air and collecting the products in a cold trap. The condensate was tested for toxicity in conventional cell cultures. No evidence was found for unique toxicity of the O_3 or air-exposed fuels compared to unexposed fuel.

In addition to using established mammalian cell lines in these studies, cell strains established from adult rat lung have been used. *These latter cell strains have been found to respond to oxidant gases in a manner similar to the established lines.*

The experimental details of these studies are presented below.

Experimental Results

Studies with Cell Line V79.

Methods

Line V79 was derived from Chinese hamster lung and is considered to be a fibroblastic cell type. The details of culture have been described previously (2,3) and will only be summarized here. Cell culture medium was obtained from commercial sources (Grand Island Biological Co.) The cells were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics.

For exposure to gases or vapors, the cells were seeded onto cellulose membrane filters (Millipore, HAWP). Cell suspensions were placed atop the filters either in culture dishes or after the filters had been assembled into the special holders. The cells were allowed to settle onto the filters and to attach. The filter holders were then placed in the exposure chambers, the chambers sealed, and gas flow through the chambers was initiated. When the desired concentration has been reached within the chambers, the medium overlying the cells was withdrawn and the exposure begun. Slow perfusion of medium through the filters was started at the same time. At the end of the exposure period, the chambers were opened, the holders removed, and the cell-bearing filters treated as appropriate to the experiment.

For measurements of cytotoxic effects of O_3 or NO_2, the filters were transfered to petri dishes containing culture medium, and returned to a 37° 5% CO_2 incubator. Cell survival was measured as the ability of cells on the filter to form macroscopic colonies after 6-7 days of incubation.

The effects of oxidant gases on DNA synthesis in V79 cells was measured as described by Painter (6). Cellular DNA was prelabeled with ^{14}C-thymidine (^{14}C-TdR) by incubating the cells for at least 2 cell generations in the presence of the tracer. After exposure to gas, the cultures were pulse-labeled (10 min.) at intervals with ^{3}H-thymidine (^{3}H-TdR), and immediately fixed with cold 5% trichloroacetic acid. Measurement of the ^{3}H/^{14}C ratio by scintillation counting gave an index of the rate of DNA synthesis at the instant of labeling with ^{3}H-thymidine.

The incidence of sister chromatid exchanges (SCEs) in V79 cells was determined essentially by the method of Perry and Wolff (7). The cells, which have a cycle time of 12 hr, were planted on filters and grown for approximately 18 hours in medium containing 10^{-5}M 5-bromodeoxyuridine (BrdUrd). At this point, the cells were exposed to O_3 at 0.035 ppm for 1 hour, and then returned to medium containing BrdUrd and colcemide (0.1 μg/ml) for an additional 3-4 hours. Thus, SCEs induced during the second replication cycle in the presence of BrdUrd would be detected.

Results

Cytotoxic Effects of O_3.

Previous studies had shown that V79 cells were very sensitive to direct exposure to O_3 as measured by colony-forming ability of cells on membrane filters exposed to the gas. Studies with radio-labeled cells indicated that the cells were disintegrating or detaching from the filters after exposure. These studies have been carried further during the present year.

Cell suspensions were seeded onto filters assembled into the holders at 2.5 x 10^5 cells per filter. Twenty-four hours later, the cell-bearing filters were exposed to 0.05 ppm of O_3 for periods of 1 to 4 hours. The filters were then removed from the holders, and treated with 0.25% trypsin to remove attached cells. The cells were collected by centrifugation, counted, an aliquot taken for viability testing with trypan blue (8), and a

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second aliquot seeded into dishes to determine colony-forming ability (CFA). The results are shown in Table 1. It is evident that exposure to O_3 caused a substantial loss in the fraction of cells recovered from the filters. Exposure to air also caused some loss, but not to the same extent. The cells recovered were nearly all viable as judged by trypan blue exclusion and the CFA was not different from cells held in immersed culture. The state of viability of the cells which detached from the filters during gas exposure could not be determined since they would presumably be carried off with the medium perfusing through the filters. These results support the previous conclusion that O_3 affects the cells in a way that causes them to detach from the filters.

Effect of O_3 on DNA Synthesis in V79 Cells.

Chemicals and physical agents which damage cellular DNA also tend to inhibit DNA replication. If the DNA damage is not rapidly repaired, the rate of DNA replication will continue to decline with time (6). There have been reports that O_3 may cause chromosomal damage (9), and may therefore cause DNA damage. Experiments to examine the effect of low levels of O_3 on DNA synthesis were done in order to obtain evidence regarding possible DNA damage by O_3.

The method was as described above. Cells were prelabeled with ^{14}C-TdR, planted on filters, and after allowing time for attachment, exposed to O_3, air, or to UV light as a positive control agent known to damage DNA. Cultures were pulse-labeled with ^{3}H-TdR at intervals following the gas or UV exposure.

The results of three experiments are summarized in Table 2.

TABLE 1

Cytotoxic Effect of 0.05 ppm O_3 on V79 Cells.

Expt. No.	Treatment	Fraction of Cells Recovered[a]	Fraction Viable[b]	CFA[c]
35-79	Immersed	1.0	1.0	0.403
	Air, 2 hr	0.70	0.95	0.309
	Air, 4 hr	0.44	0.84	0.30
	O_3, 2 hr	0.12	0.92	0.26
	O_3, 4 hr	0.065	0.89	0.42
37-79	Immersed	1.0	0.99	0.28
	Air, 1 hr	0.22	0.87	0.44
	Air, 3 hr	0.36	0.88	0.26
	O_3, 1 hr	0.072	0.78	0.22
	O_3, 3 hr	0.080	0.94	0.25

[a] Compared to immersed control

[b] Fraction of recovered cells indicated as viable by trypan blue exclusion (8).

[c] Colony-forming ability of cells indicated as viable by trypan blue exclusion expressed as a fraction of cells plated. Values are based on five replicate plates.

TABLE 2

Effect of O_3 Exposure on the Rate of DNA Synthesis in V79 Cells.

Expt. No.	O_3 Dose	Ratio O_3/Air[a]	Ratio UV/PBS[a]	^{14}C-DPM on O_3-exposed Filter[b]
1-80	0.03 ppm, 1 hr	0 hr = 0.271	0 hr = 0.95	Air, 0 hr = 6756
		4 hr ≈ 0.34	4 hr = 0.84	O_3, 0 hr = 3810
				Air, 4 hr = 6403
				O_3, 4 hr = 3329
2-80	0.03 ppm, 1 hr	0 hr ≈ 0.35	0 hr = 0.68	Air, 0 hr = 3048
				O_3, 0 hr = 2299
		3 hr ≈ 0.84	3 hr = 0.71	Air, 3 hr = 1635
				O_3, 3 hr = 1642
		4 hr = 1.57	4 hr = 0.44	Air, 4 hr = 510
				O_3, 4 hr = 2016
4-18-80	0.035 ppm, 1 hr	0 hr = 0.87	0 hr = 0.89	O_3, 0 hr = 2863
		0.5 hr = 0.89	0.5 hr = 0.90	O_3, 0.5 hr = 2061
		1.5 hr = 0.54	1.5 hr = 0.69	O_3, 1.5 hr = 593
		3.5 hr = 1.91	2.5 hr = 0.59	O_3, 3.5 hr = 408
			3.5 hr = 0.69	

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[a]Calculated as the ratio of ^{3}Hdpm/^{14}Cdpm in the O_3 or UV exposed cells divided by the ^{3}Hdpm/^{14}Cdpm ratio in the corresponding control.

[b]Total ^{14}Cdpm remaining on filter at the time of measurement. Values are the mean of 3 or 4 filters.

One effect of the O_3 exposure was to cause a loss of ^{14}C-labeled cells from the filters. This finding confirms previous observations which indicated cells were being lost after O_3 exposure.

In most experiments, O_3 produced a decrease in the rate of DNA synthesis which persisted for some time after exposure, but eventually returned to the control level or above. Exposure to UV light also caused a reduction in the rate of DNA synthesis, which

did not recover to control levels during the time period studied, and in some experiments, the rate of DNA synthesis continued to decline. The conclusion from these studies is that exposure of cells to O_3 under the conditions described did not produce DNA damage to an extent that would inhibit DNA synthesis. The pattern of inhibition of DNA synthesis by O_3 resembled that seen after exposure of cell cultures to toxins that are metabolic poisons which do not damage DNA (6). The data also suggest that if cells are not lost from the filters, they will recover from the effects of the O_3 exposure. This latter conclusion is supported by the experiments in which cytotoxic effects were examined using trypan blue dye exclusion tests and colony-forming ability.

A Test for Sister Chromatid Exchange (SCE) Induction by O_3.

The results of tests for SCE induction in V79 cells by O_3 showed no significant difference between the SCE incidence in air-exposed and O_3-exposed cells. The data in Table 3 are from 2 independent counts of the SCE incidence. The graphs of one of these sets of data are shown in Figure 8. Although the data are suggestive of a higher incidence of SCEs in the O_3-exposed cells, a firm conclusion cannot be made.

TABLE 3

Test for Sister Chromatid Exchange Induction in V-79 Cells by Ozone.

Cell cultures were exposed on filters to 0.03 ppm of O_3 for 2 hours. The cells had been grown in medium containing BrdUrd (10^{-5} M) for 18 hours prior to exposure to O_3. Following exposure, the cell-bearing filters were transferred to culture dishes with medium containing BrdUrd for an additional 4 hours at which time the cells were harvested and stained for SCE analysis as described (7). The values are SCEs per cell $\pm$ S.D. The numbers in parentheses are the number of cells counted. Only complete metaphases were scored. The slides were scored twice with different observers.

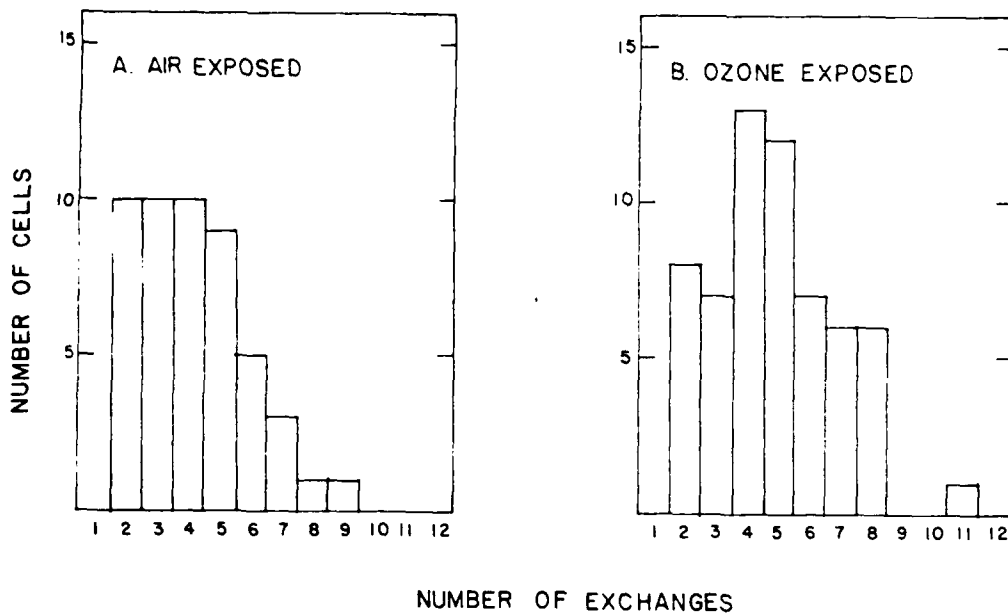|	O_3-Exposed	Air-Exposed
Count #1	6.6 $\pm$ 2.2 (53)	5.1 $\pm$ 1.7 (60)
Count #2	4.87 $\pm$ 1.98 (60)	4.05 $\pm$ 1.62 (60)

FIGURE 8. Sister chromatid exchange incidence in cell line V-79 after exposure to ozone for 1 hour at 0.035 ppm.

Effects of NO$_2$ on DNA Synthesis in V79 Cells.

Figure 9 shows the results of studies on the survival of CFA of V79 cells after exposure to 0.15 ppm of NO$_2$ for the times indicated. These data confirm previous findings of the great sensitivity of V79 cells to inhibition of colony formation by NO$_2$. The basis for this inhibition is probably the same as for the effects seen with O$_3$, i.e., cells are being destroyed or otherwise lost from the filters as a consequence of the NO$_2$ exposure.
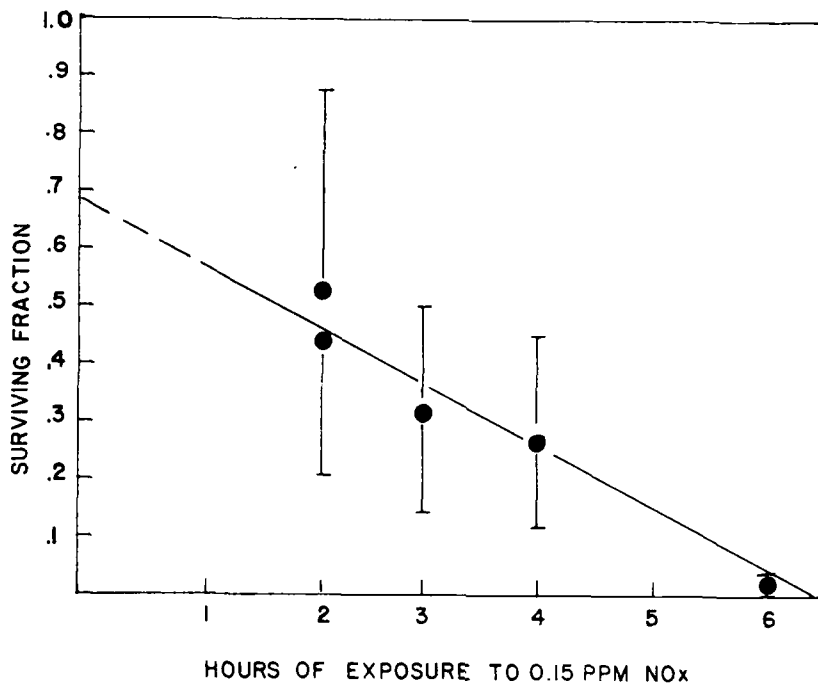
FIGURE 9. Cytotoxic effect of NO_2 on V-79 cells. The data points indicate the survival of colony forming ability relative to cells exposed to clean air for the same time period.

Previously reported preliminary studies suggested that NO_2 may have had an inhibitory effect on DNA synthesis in V79 cells. Further studies have shown that, at NO_2 doses which do not result in extensive cell loss, there is a transient inhibition of DNA synthesis followed by recovery. In several cases, the rate of DNA synthesis in the NO_2-exposed cultures exceeded that in the air-exposed. The data from these studies are summarized in Figure 10. The results of positive-control studies with UV light were as

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expected in that the rate of DNA synthesis continued to decline after the short exposure to UV light. In the cultures exposed to 0.15 ppm of NO_2 for 1 hour, there was no evidence of significant cell loss from the filters as judged by comparison of the ^{14}C label remaining on the filters between NO_2 and air-exposed cultures.

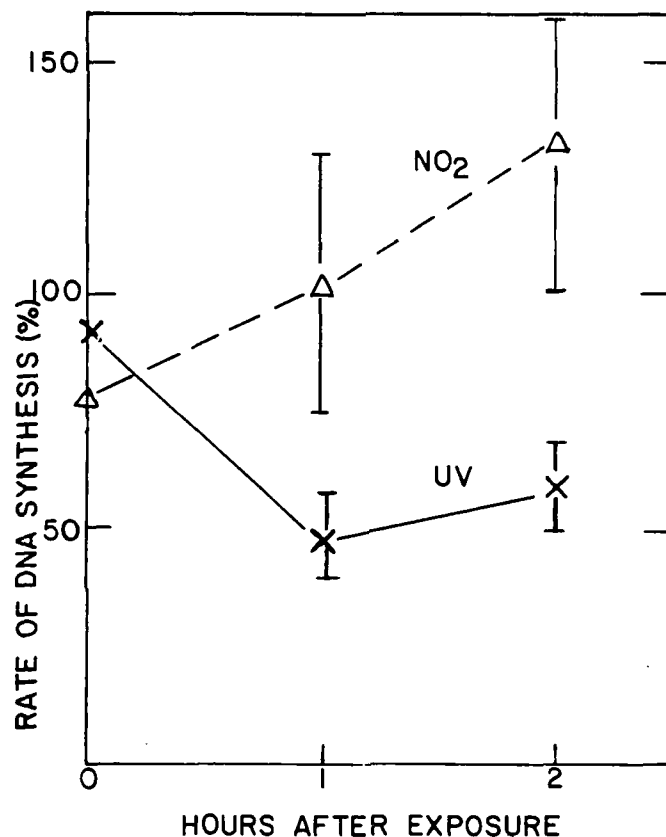FIGURE 10. Effect of ultraviolet light or NO_2 on the rate of DNA synthesis in V-79 cells measured immediately after exposure and at 1 and 2 hours later. The values are plotted as a percentage of the rate of DNA synthesis in corresponding control cultures.

The conclusion from these studies is that exposure to 0.15 ppm of NO_2 for 1 hour did not cause DNA damage sufficient to inhibit DNA synthesis.

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Test for Toxic Products of Reaction Between O_3 and J P-5.

Using the fuel vaporization method described above, JP-5 vapors were mixed with O_3 in air. The ozone concentration was between 0.7-0.8 ppm. The O_3 concentration could not be measured in the presence of the fuel vapors because the vapors fouled the O_3 measuring equipment (Dasibi 1003AH) which depends on ultraviolet absorbance of the gas. Therefore, it was not possible to determine the loss of O_3 upon mixing with the JP-5 vapor.

The condensed fuel vapor was dissolved in DMSO at 100 mg/ml, and aliquots of this solution were used to prepare solutions of fuel in PBS (pH 7.2-7.4) at 1 mg/ml. Cells grown in glass culture flasks were exposed to the fuel-PBS for 1 hour. After exposures the cells were suspended by trypsinization, viability determined by trypan blue exclusion, and aliquots plated for colony-forming ability at 200 viable cells per 60 mm dish. The data obtained in 3 experiments are shown in Table 4. Because of the limited nature of this study, only preliminary conclusions are possible. They are:

1. JP-5, treated with air, O_3, or not treated, is toxic to cells in culture to similar extents;

2. Although cells treated with JP-5 may appear viable by the trypan blue exclusion test, they may be unable to form colonies;

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TABLE 4

Cytotoxic Effects of Aircraft Fuel JP-5 on Cell Line V-79.

Fuel Sample	Viability		CFA (Colonies/Viable Cells Planted)[a]
Petroleum JP-5	Medium Control	1.0	0.52
	Medium + DMSO	1.0	0.51
	PBS + DMSO	1.0	0.46
	PBS + JP-5(Air)	0.37	0.06
	PBS + JP-5(O_3)	0.28	0.06
Petroleum JP-5	Medium Control	1.0	0.35
	Medium + DMSO	0.99	0.46
	PBS + DMSO	0.98	0.66
	PBS + JP-5(Air)	0.84	0.01
	PBS + JP-5(O_3)	0.92	0.37[b]
	PBS + JP-5(N.T.)[c]	0.84	0.005
Shale JP-5	Medium Control	0.99	0.51
	Medium + DMSO	0.99	0.46
	PBS + DMSO	0.99	0.42
	PBS + JP-5(Air)	0.77	0.035
	PBS + JP-5(O_3)	0.87	0.02
	PBS + JP-5(N.T.)	0.16	0.08

[a]Viability was determined with trypan blue method (8). CFA was determined on the basis of 5 replicate plates seeded with 200 viable cells per plate.

[b]Aberrant value; possible dilution error in planting cells.

[c]N.T. = Not exposed deliberately to air or ozone.

SECTION III. Evaluation of Rat Lung Cell Strains for Use in Cell Exposure System

Introduction and Summary

The isolation of cell strains from rat lung was undertaken in order to provide cell types which retain functional or morphological features of cells in vivo. These strains would then be used in the cell exposure system. Two cell strains, designated ARL-12 and ARL-14, have been isolated from adult rat lung by adaptation of published procedures. The cells have an epitheloid appearance, and have some characteristics which suggest that they are derived from type 2 alveolar cells. They have been maintained in continuous culture since March 1978. Although the cell strains resemble each other, they do have certain differences. The chromosome numbers are different with ARL-12 having a modal number of 44 and ARL-14 having 43. The cell cycle times in culture are similar as are the growth requirements. Slight differences between the strains have been seen in the metabolic conversion of benzo(a)pyrene (BaP) to hydroxylated derivatives.

Both cell strains will attach and grow on the Millipore membrane filters used in the exposure system. The sensitivity of the cell strains to O_3 was similar to that of cell line V-79. Ozone inhibited DNA synthesis in both strains, but studies have provided no evidence for a direct effect on cellular DNA. Tests for SCE induction in strain ARL-14 by O_3 were negative.

Methods

Isolation of Cell Strains.

The methods used were adapted from Mason, et al. (10) and Kikkawa (11). Adult male Sprague-Dawley rat lungs were washed free of blood by perfusion via the pulmonary artery, and alveolar macrophages were lavaged from the lung via the trachea using a HEPES-buffered salt solution without Mg^{++} or Ca^{++} (10). The lungs were inflated with a 0.25% trypsin solution, and, following a period of incubation, they were minced and

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shaken to release the cells freed by the trypsin treatment. The cells were further purified by filtration through a nylon mesh and centrifugation (1 hr 1700 x g) in a discontinuous Ficoll gradient. The cells banding at the interface between densities 1.040 and 1.080, as well as those at the bottom of the tube, were collected separately, washed with F12K medium (12) and seeded into culture flasks (Falcon, 25 cm^2). After 3 hours, the supernatant fluid was removed to a new flask and the attached cells in the first flask discarded, the rationale being that fibroblasts and macrophages would attach quickly, whereas the epithelial cells would not. Therefore the second flask would be enriched in epithelial cells relative to other types. After a further 20 hours of incubation, the growth medium was renewed and any floating cells discarded. Only the cells recovered from the pellet gave rise to cultures containing significant numbers of epithelial type cells, and it was from these cultures that the clones of strains ARL-12 and ARL-14 were eventually isolated. The methods followed for cloning have been published (13).

In culture both strains have an epitheloid appearance. Inclusion bodies resembling those seen in type 2 alveolar cells are present. Living cells stained with phosphine dye show fluorescent cytoplasmic granules as reported by others (10) who have studied similar cells in culture. The cells show "contact inhibition" of growth and form a continuous monocellular layer without piling up or overgrowth.

The cells were originally grown in medium F12K (12) supplemented with 10% fetal bovine serum. Subsequent tests indicated that better growth, in terms of rate of proliferation, was obtained with Waymouth's MB752/1 medium, also with 10% fetal bovine serum. Antibiotics used were penicillin, 100 units/ml; streptomycin 100 µg/ml, and Fungizone, 20 µg/ml. Additional growth factors tested were insulin (1 µg/ml), neuraminidase (1 unit/ml), and hydrocortisone (3 x 10^{-6} M). Only hydrocortisone appeared to enhance growth, and then only slightly. None of these growth factors have been used routinely in the experiments.

Exposure of ARL Cells to Gases.

The methods were essentially the same as described above for V-79 cells. Cells were seeded onto filters assembled into holders, or onto filters in culture dishes as appropriate. For tests of the induction of SCEs in ARL-14 by O_3, the cells were grown in glass culture flasks which were fitted with stoppers having tubing connections to permit injection of gases. The effluent gas from the flasks was directed to the Dasibi O_3 monitor for analysis.

Measurement of Benzo(a)pyrene (BaP) Metabolism by ARL Cells.

Cells of strains ARL-12 and ARL-14 were seeded into 150 mm culture dishes at a density of approximately 1.4×10^4 cells/cm^2. Twenty-four hours later, the culture medium was renewed with fresh medium containing a known inducer of the P-450 enzymes responsible for BaP hydroxylation (14,15). The inducers included 2, 5-diphenyloxazole (PPO), beta-naphthoflavone (BNF), and phenobarbital (PB). The final concentration of all inducers was 3×10^{-5} M. For solution in the medium, PPO and BNF were first dissolved in dimethylsulfoxide (DMSO); PB was dissolved directly in medium. Control cultures received DMSO (final concentration 1%) or no additions. For comparative purposes, cultures of a strain of rat tracheal epithelial cells, isolated in this laboratory, were given similar treatment. At 48 hours after adding the inducers, the cell cultures were harvested by trypsinization, counted, and 10^7 cells incubated at 37° for 4 hours in 1 ml fresh medium which contained 10 μCi of ^{3}H-BaP (10 Ci/mmole). At the end of the incubation period, cells and medium were extracted twice with 2 ml portions of ethyl acetate. The organic extracts were pooled, evaporated to near dryness under N_2, and the residue chromatographed on thin-layer silica gel plates using benzene and benzene:ethanol (19:1) as solvents as described elsewhere (16). Individual metabolites of ^{3}H-BaP were detected by fluorescence under UV light and by scanning of the plates with a radiochromatogram scanner. Radioactivity associated with the metabolites was

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quantified by scintillation counting of regions cut from the plates which carried the individual metabolites. Enzymatic activity was calculated as pmoles of metabolite produced per mg cellular protein during the 4-hour incubation.

Visualization of Intracellular Inclusions with Phosphine Dye.

A stock solution of phosphine dye (K & K Laboratories, Irvine, CA) was made at 2 mg/ml in DMSO. Ten µl of this solution was added to 10 ml of 0.9% NaCl for use. Equal volumes of the dye solution and cell suspension in growth medium were mixed and allowed to stand for 5-10 min. Drops of the suspension were placed on microscope slides and immediately examined with a Nikon microscope with fluorescence attachments.

Survival of ARL Cells After Exposure to UV Light.

Cells were seeded into 60 mm plastic dishes and allowed 3 hours for attachment. The growth medium was then removed and replaced with 2 ml of PBS. The uncovered dishes were exposed to UV light from a 4 watt low pressure Hg lamp (Mineralight) at a dose rate of approximately 1.0 J/m^2 for 5, 10, 15 or 20 sec. Controls included cells exposed to PBS and cells held in growth medium. After UV exposure, the PBS was replaced with growth medium and the dishes incubated for 11 days, at which time they were stained and macroscopic colonies of 50 cells or more were counted.

Results

Morphological Characteristics of Cell Strains.

The cell cultures obtained from trypsin treated lungs at first consisted of several cell types that, in general, could be classified as epitheloid or fibroblastic types. When the cells were subcultured and allowed to regrow in dishes, the predominant cell type was epitheloid, but the cells appeared vacuolated and "unhealthy". With further feeding, areas of cell growth appeared which contained both epithelial and fibroblastic types, and

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which appeared to be capable of replication. A mixed population from one of these areas was transferred to a 25 cm^2 culture flask. These cells were carried through 6 subcultures, at which point the cells were seeded into 100 mm dishes at 1000 cells per dish. When colonies had formed, the dishes were examined microscopically, and several colonies which appeared to be epithelial-like were transferred individually to Leighton tubes. When the selected populations had proliferated to fill a 75 cm^2 flask, they were cloned a second time. The clones finally selected and designated ARL-12 and ARL-14 were chosen on the basis of morphologic markers.

The two clones are similar in that both show epitheloid growth in culture, and show density inhibition of growth. They both contain cytoplasmic inclusions which are stainable with fluorescent phosphine dye. They differ in chromosome number, and possibly other biochemical activities (see below).

At this writing, the strains have been in continuous culture since March, 1978, and show no sign of degeneration. They are passaged at weekly intervals, usually with a 1:10 split ratio. Early passages have been preserved in liquid N_2 for future reference.

UV Sensitivity of ARL Cells.

The survival of colony-forming ability (CFA) of the ARL cells after exposure to UV light was determined in order to compare their response with that of other cell lines. Figure 11 is a plot of the survival curves obtained. Strain ARL 12 appears slightly more sensitive to UV light than ARL-14. In experiments with other cell lines, a slight shoulder is observed, as with strain ARL-14. This experiment suggests that these strains may be useful in studies of factors influencing radiation sensitivity such as DNA repair capabilities.
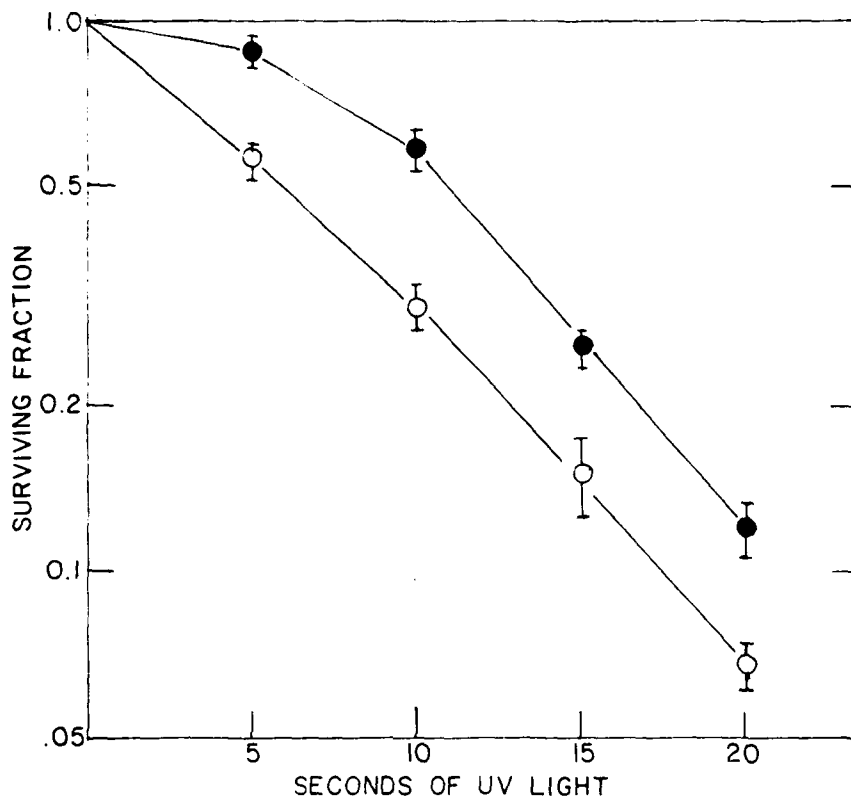
FIGURE 11. Survival of colony forming ability in strains ARL-12 and ARL-14 after exposure to ultraviolet light. The dose rate was approximately 1 $j/m^2 sec^{-1}$. Data points are the mean of 5 replicate plates ± 1 S.E., and are plotted as the surviving fraction relative to unexposed control cultures. Filled symbols, ARL-14; open symbols, ARL-12.

BaP Metabolism by ARL Cells.

The results of this study are shown in Table 5. The major findings were that both strains of ARL cells were able to convert BaP to mono- and dihydroxylated derivatives, but the enzymatic activity could not be increased by pretreatment of the cells with known inducers of BaP-metabolizing enzymes. Studies with a strain of epitheloid cells derived from rat trachea showed that the enzymes could be induced under the culture conditions employed. Only BNF showed significant inducer activity in the tracheal cell strain. Published reports have indicated that PPO is an inducer in some cell strains (15), but it showed little activity in the tracheal or lung cells used in these experiments. Phenobarbital (PB) has been found to be active as an inducer only in liver in vivo or in liver cells in culture (17).

The metabolite profile was different for each of the cell strains. The most prominent metabolites produced by ARL-14 cells were the 7, 8- and 9, 10-dihydrodihydroxy (diol) derivatives of BaP, while with ARL-12 and the tracheal cells, the monohydroxy derivatives were predominant. This difference in metabolite profiles may be due to different P-450 enzymes being produced by the different cell strains. If so, these strains might be useful in studies of the genetic or biochemical control of the synthesis of this important class of enzymes.

TABLE 5

Benzo(a)pyrene Metabolism by ARL Cells and Rat Tracheal Cells in Culture.

Cell cultures were treated with the inducers of benzo(a)pyrene hydroxylating enzymes and harvested as described in the text. The inducers were dissolved in DMSO and added to the cell culture medium 48 hours before harvest. The values are pmoles of metabolite/mg protein produced during a 4-hour incubation.

| Metabolite | DMSO-Treated[c] | | | | PPO-Treated | |
	ARL-12	ARL-14	Tracheal	ARL-12	ARL-14	Tracheal
9,10-diol[a]	27	93	76	29	136	93
7,8-diol	20	47	76	22	69	48
4,5-diol	2	2	5	1	3	10
BP-OH[b]	33	39	187	35	37	220

| | BNF-Treated | | | | PB-Treated | |
	ARL-12	ARL-14	Tracheal	ARL-12	ARL-14	Tracheal
9,10-diol	9	98	102	20	84	77
7,8-diol	10	57	110	16	41	73
4,5-diol	31??	2	6	2	1	4
BP-OH	36	59	447	26	38	177

[a]diol=Dihydrodihydroxy-BaP

[b]BP-OH contains 3-hydroxy-Bap and 9-hydroxy-BaP.

[c]DMSO=dimethylsulfoxide; PPO=2,5-diphenyloxazole; BNF=beta-naphthoflavone; PB=phenobarbital. The final concentration of all inducers was 3×10^{-5} M.

Sister Chromatid Exchange in ARL-14.

Cultures of ARL-14 were exposed to O_3 as described above and prepared for SCE analysis (7). The slides were scored blind, and only complete metaphases were counted. the results (Table 6) showed no difference between exposed and control cultures.

TABLE 6

A Test for Induction of Sister Chromatid Exchanges in ARL-14 Cells.

Values are SCEs per cell ± S.D. Only complete metaphases were scored. The number in parentheses is the number of cells scored.

Ozone-Exposed, 0.25 ppm, 2 hr	Air-Exposed, 2 hr
14.9 ± 3.9 (56)	14.1 ± 4.6 (70)

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