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LA-7680-PR

Progress Report

DR 2349

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**Detection of Early Changes
in Lung Cell Cytology by
Flow-Systems Analysis Techniques**

January 1—December 31, 1978

University of California



LOS ALAMOS SCIENTIFIC LABORATORY

Post Office Box 1663 Los Alamos, New Mexico 87545

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This project received support from the US Department of Energy and the US Environmental Protection Agency, LASL Project R-250, EPA Agreement EPA-IAG-D5-E681.

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UNLIMITED

DETECTION OF EARLY CHANGES IN LUNG CELL CYTOLOGY

BY FLOW-SYSTEMS ANALYSIS TECHNIQUES

by

J. A. Steinkamp, J. S. Wilson, Z. V. Svitra, and K. M. Hansen

ABSTRACT

This report summarizes ongoing experiments to develop cytological and biochemical indicators for measuring damage to respiratory tract cells of experimental animals exposed by inhalation to environmental toxic agents. The specific goal is to apply flow cytometric methods to analyze and detect changes in lung epithelium as a function of exposure to toxic agents associated with the production and utilization of synthetic fuels from oil shale and coal. During the past 6 months, hamsters were exposed to raw and spent oil shale particulates, silica dust, and ozone, and DNA content measurements were performed on lung cell samples. Although initial shale exposures did not yield the expected results, recent data show atypical changes in DNA content per cell distributions. Ozone exposures also were expanded to include DNA measurements and cytology, ranging up to 72 h post-exposure. Progress was achieved in developing a new method for quantitating pulmonary macrophage phagocytosis in rats using micron-sized fluorescent spheres. New methods for determining alkaline phosphatase, DNA content, and protein also were under development. Plans are to continue developing cytological and biochemical markers for measuring atypical cellular changes, including macrophage function, and to emphasize exposing experimental animals to particulates and gaseous agents for studying dose-damage relationships.

I. INTRODUCTION

The objective of this health effects program is the application of modern flow cytometric analysis techniques¹⁻³ to develop analytical methods for assaying cytological and biochemical damage to respiratory tract cells in experimental animals exposed (inhalation) to toxic agents associated with the production and utilization of synthetic fuels from fossil resources such as oil shale and coal.⁴⁻⁸ This technology provides a new approach for assessing damage to respiratory tract epithelium and pulmonary macrophages, the end objective being examination of human sputum samples. The final output ultimately may serve to provide assistance in estimating risks, evaluating incipient damage, and establishing guidelines for determining exposure levels of various toxic agents to occupationally exposed populations and society-at-large.

To develop the analytical techniques necessary for quantitative assessment of cellular damage in animal models exposed to physical and chemical toxic agents, recent efforts have been directed toward (1) the application of automated cell analysis and sorting instrumentation, including staining methods, to measure atypical changes in DNA content distributions of exfoliated lung cells from Syrian hamsters exposed to oil shale particulates, silica, and ozone; (2) the development of a new approach for assaying pulmonary macrophage phagocytosis using polystyrene latex fluorescent microspheres; and (3) the investigation of some new methods for measuring alkaline phosphatase, DNA content, and total protein. Improvements to the dual-laser cell analysis system^{8,9} included the addition of a second high-power laser (argon) to be used in conjunction with the present krypton laser

for single- or dual-laser excitation of fluoro-chromes, with absorptions ranging from the uv to the ir.

II. MATERIALS AND METHODS

To study cellular changes in animals exposed to particulates of oil shale and silica, 24 Syrian hamsters were divided into 6 experimental groups. The first group (a total of 3) was used as controls. A second group (a total of 3) was instilled (intratracheally) with 0.2 ml of normal saline. The remaining groups, consisting of 6 animals each, were injected intratracheally with 10 mg, respectively, of ball-milled (2- to 7- μ m-diameter range) raw and spent shale particulates and silica suspended in 0.2 ml of saline. Raw shale (Type 2) was obtained from Anvil Points, Colorado. The two spent shales (Types 1 and 2) were from solid heat transfer and gas combustion processes (facilities), respectively. Silica was a commercial product obtained from the Pennsylvania Glass and Sand Corporation, having a mean diameter of 4 μ m. The hamsters were anesthetized with "Brevital" (5 mg) for intratracheal instillation via the oral cavity. All animals were returned to the colony (with 1 animal from the control and saline-instilled group and 2 animals from the oil shale particulate and silica groups) and sacrificed 28, 35, and 42 days later. The lungs were then lavaged 4 times with saline to obtain exfoliated cells, which were then centrifuged, rinsed in saline, and fixed in 35% ethanol prior to staining for DNA content with mithramycin,^{10,11} exciting at 457 nm (argon laser), and analyzing for fluorescence properties.

The hamsters were exposed also to acute levels of ozone (4 ppm for 4 h) and sacrificed at different times, ranging from 0 to 72 h after termination of exposure. Respiratory tract cells were obtained at sacrifice using pentobarbital, followed by lavaging the lungs with saline, fixing in 35% ethanol, and staining with mithramycin (DNA content) for analysis.

To begin development of an automated method for quantitating pulmonary macrophage phagocytosis, normal Sprague-Dawley rats were anesthetized by inhalation of Metaflane. The trachea was then intubated with an 18-gauge blunt needle via the oral cavity, and 1 to 2 x 10⁷ polystyrene latex spheres (fluorescent) of 1.83- μ m diameter suspended in

0.5 ml of saline were delivered to the respiratory tract. After 2 h, the rats were sacrificed by pentobarbital injection and their lungs lavaged with 4 ml of normal saline (4 times). Cells were fixed in 35% ethanol, resuspended in saline, excited at 457 nm (argon laser), and analyzed for fluorescence (phagocytized spheres) and light scatter (size). Slide preparations and differential counts also were made of the samples.

To continue development of new staining methods for cellular biochemical parameters, model cell lines were stained and analyzed for DNA, protein, and alkaline phosphatase. DNA content and protein have been stained recently with propidium iodide (PI) and bis-[4-aminophenyl]-1,3,4-oxadiazol (BAO), respectively.¹² Both PI and BAO are excitable in the uv and have emission peaks near 430 and 625 nm, respectively. Model cell lines, including normal hamster and rat respiratory tract cells, were fixed in ethanol, treated with RNase, and analyzed for DNA and protein using two-color fluorescence analysis methods.¹²

Preliminary studies using naphthol AS-BI as a fluorescent substrate for measuring alkaline phosphatase activity in respiratory tract cells also were initiated. Normal hamsters and rats were sacrificed using "Brevital" and the lungs lavaged with normal saline. Cells were fixed in glutaraldehyde, reacted with naphthol AS-BI, and analyzed for fluorescence.

Normal and exposed respiratory tract cell samples composed of macrophages, leukocytes, ciliated columnar and basal undifferentiated cells stained with fluorescent dyes were analyzed in liquid suspension as they flowed through a chamber intersecting a laser beam(s) of exciting light (i.e., mithramycin--457-nm line, 1.83- μ m-diameter fluorescent microspheres; BAO, PI, and naphthol AS-BI--uv lines).^{9,13} Multiple sensors measured the fluorescence and light-scatter optical signals on a cell-by-cell basis. Cellular parameters proportional to optical measurements (e.g., DNA content, phagocytic activity, cell size, protein, and alkaline phosphatase activity) were displayed as frequency distribution histograms using a multi-channel pulse-height analyzer. Cells also were separated according to various cytologic parameters and identified microscopically.

III. RESULTS AND DISCUSSION

During this report period (January 1 to December 31, 1978), major emphasis was placed on exposing test animals to oil shale particulates and ozone and on developing new methods for measuring pulmonary macrophage phagocytosis, DNA, protein, and alkaline phosphatase activity. However, an important improvement was made to the dual-laser excited multiparameter cell analysis and sorting instrumentation (see Fig. A-1) which has direct application to this program. A new high-power argon-ion laser with additional output capability in the uv was purchased through an ongoing National Cancer Institute/Department of Energy interagency agreement and has been recently incorporated into the cell separator system, thus permitting extended single- or dual-laser excitation of fluorochromes bound to cells.⁹ The new argon laser (Coherent Model CR-10) replaces an older and smaller argon unit (Coherent Model CR-3) that had no uv capability. When combined with the krypton laser (see Table A-I), the Model CR-10 will add improved versatility to the dual-laser excitation system. For example, it will be possible to excite fluorescent dyes such as Hoechst or DAPI (uv excitation, argon laser) and the rhodamines (yellow excitation, krypton laser) for measuring DNA content and protein, respectively. Other fluorochrome combinations, including red and green fluorescent microspheres (i.e., phagocytosis quantitation), also may be excited using this new laser arrangement.

A. DNA Measurements: Respiratory Tract Cells

Exposed to Oil Shale Particulates and Silica Dust

To continue pilot studies with classes of crude products, particulates, and known toxic agents, hamsters were exposed to raw and spent oil shale particulates and silica by intratracheal injection. Previous results⁷ (cytology) showed a definite increase in total numbers of macrophages, leukocytes, and multinucleated cells, with variation in differential cell counts, from rats and hamsters up to 28 days after exposure to raw and spent oil shale particulates. Basal undifferentiated and ciliated columnar cells remained constant--near zero. DNA content distributions showed no significant changes compared to controls. Therefore, it was decided to examine respiratory tract cells 28 days or more

after exposure. Figure 1 shows the DNA content per cell distributions of respiratory tract cells from hamsters exposed to saline, silica, and raw and spent oil shale particulates at 28, 35, and 42 days after exposure. DNA content distributions of normal "control" animals are shown in Fig. 1A. Peak 1 represents cells having 2C DNA content and peak 2 binucleated cells and doublets (4C DNA content).⁸ The DNA content distributions of respiratory tract cells from hamsters exposed to saline (Fig. 1B) closely resemble the controls. However, DNA content distributions of respiratory tract cells from hamsters exposed to silica (Fig. 1C), which appear nearly normal at 28 days postexposure, begin to show atypical changes at 35 and 42 days. A third region has appeared to the left of peak 1, which is most likely dead cells. At 42 days, cells within region 3 have increased and a shoulder is beginning to develop on the right side of peak 1. Also, the percentage of binucleated cells appears to be increasing. Preliminary DNA content distributions of respiratory tract cells exposed to raw and spent oil shale are shown in Figs. 1D, 1E, and 1F. Figure 1D illustrates DNA content distributions of respiratory tract cells exposed to Type 1 spent shale. These distributions appear nearly normal, with the exception that the left side of peak 1 is skewed. This probably represents dead cells but has not been verified.

DNA content distributions from respiratory tract cells exposed to Type 2 raw and spent shale are shown in Figs. 1E and 1F, respectively. Distributions from hamsters exposed to raw shale appear nearly normal; however, DNA content distributions from hamsters exposed to spent shale showed atypical changes 35 to 42 days postinstillation. A definite shoulder appeared on the right side of peak 1, and cells within region 3 increased. These changes were better observed by increasing the amplifier gain of the fluorescence channel, thus centering peak 1 in channel 30 of the multichannel pulse-height analyzer (Fig. 2). Peak 1 shows a well-defined second region of cells to the right side of the DNA content distributions that is similar to results from hamsters exposed to ozone, as described below. In this set of particulate exposures, slides were made for differential cell counts, but the results are not included because

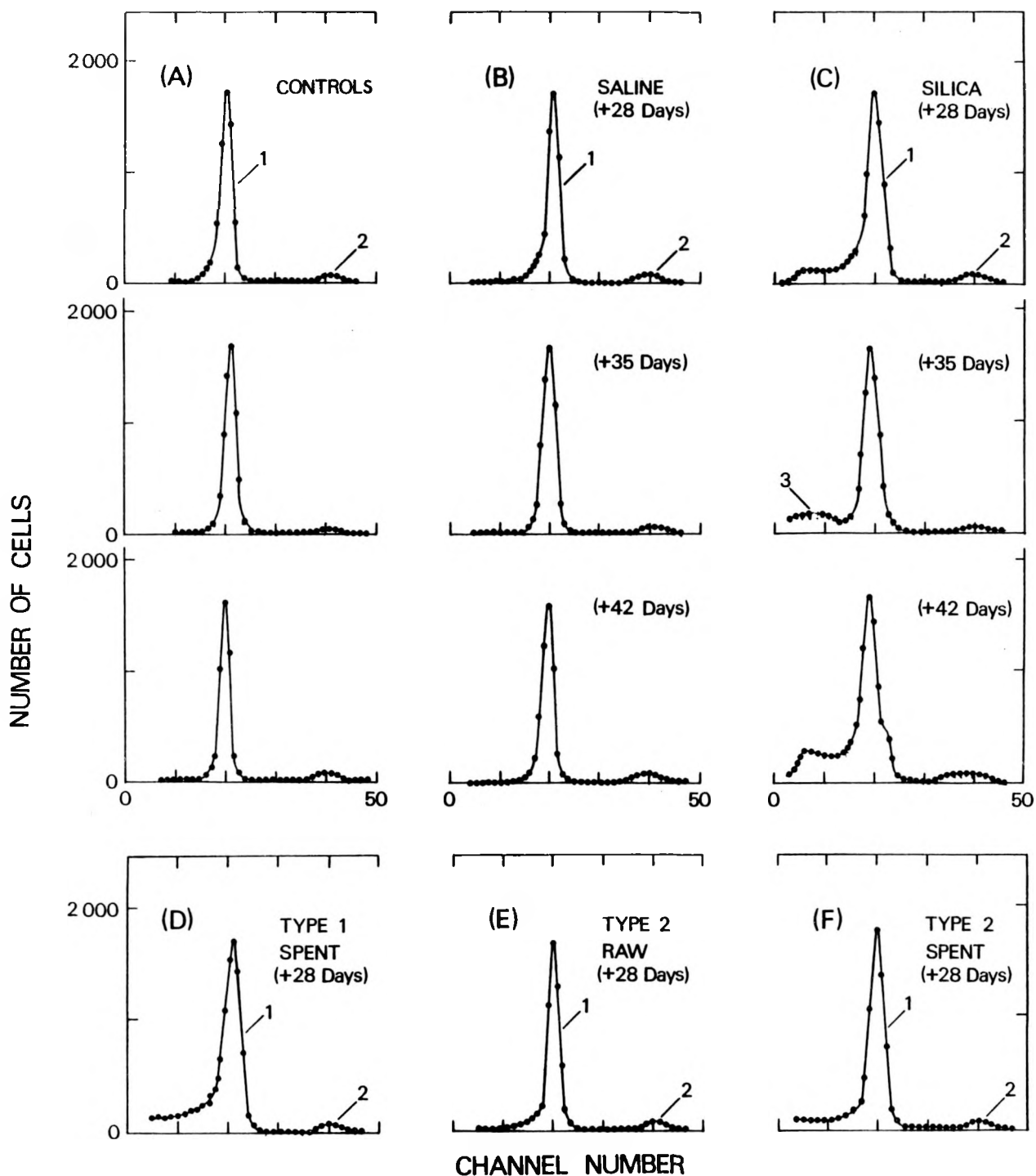


Fig. 1. DNA content frequency distribution histograms of hamster respiratory tract cells exposed (intra-tracheal injection) to saline, silica, Type 1 spent shale, and Type 2 raw and spent shale prior to sacrificing 28, 35, and 42 days later. Cell samples were obtained by lung lavage, fixed in 35% ethanol, stained with mithramycin, and analyzed for fluorescence. Types 1 and 2 spent shales were obtained from solid heat transfer and gas combustion processes, respectively.

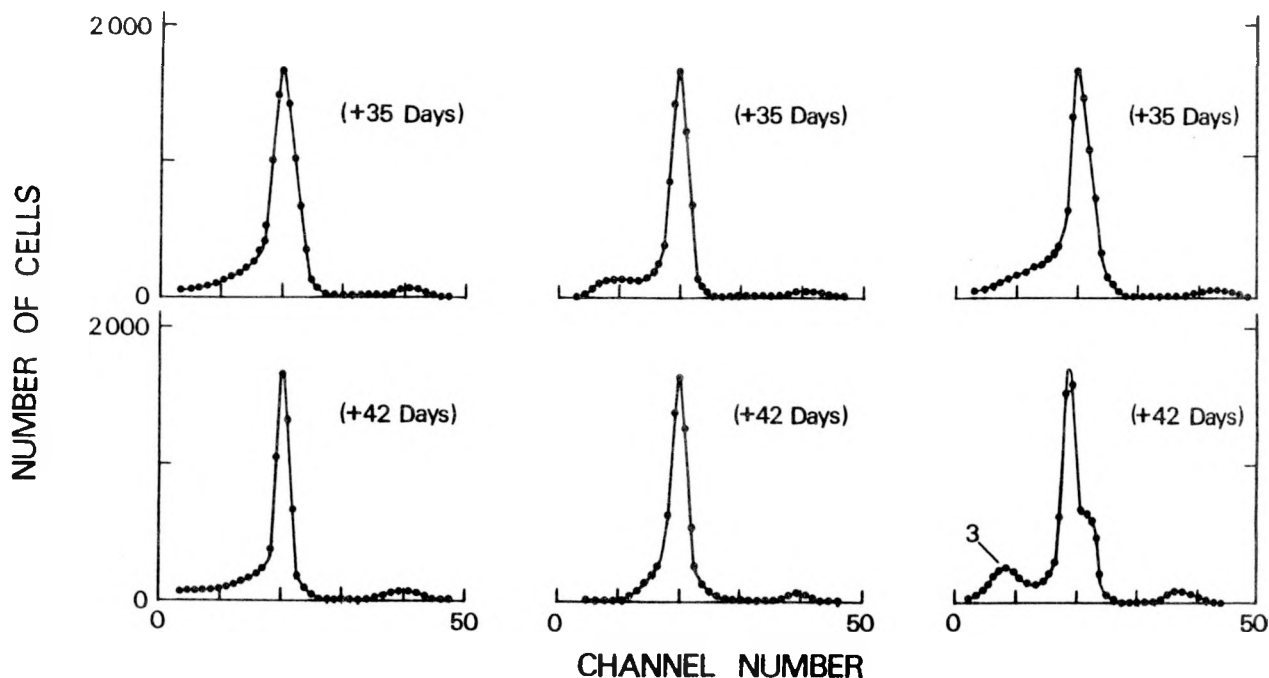


Fig. 1 (contd.).

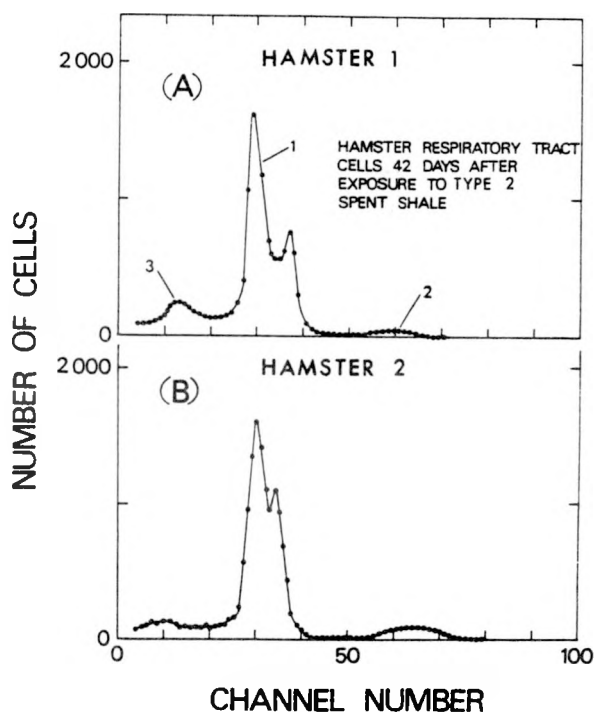


Fig. 2. DNA content frequency distribution histograms of hamster respiratory tract cells exposed (intratracheal injection) to Type 2 spent shale prior to sacrificing 42 days later. Cell samples were obtained by lung lavage, fixed in 35% ethanol, stained with mithramycin, and analyzed for fluorescence. Type 2 spent shale was obtained from a gas combustion process.

the cytology is not complete. Although not understood at this time, these preliminary data indicate that atypical changes can be detected using flow cytometric analysis methods for DNA content measurement. Further studies will concentrate on continued exposure of animals to particulates and identification of cell types associated with cell sorting and cytology. Attempts will also be made to improve resolution of the DNA measurements.

B. DNA Measurements: Respiratory Tract Cells

Exposed to Ozone

Hamsters also have been exposed to acute levels of ozone (4 ppm for 4 h). These preliminary results (unpublished program review) are shown in Fig. 3. Corresponding differential cell counts are contained in Table A-II. Figure 3A shows a DNA content distribution from a normal control hamster as described also in other reports.^{5,6,8} Similar DNA content distributions were obtained 0 and 1 h after release from exposure (Figs. 3B and 3C). Peaks 1 and 2 both show a general broadening, with an increase in percentage of cells contained in peak 2 (binucleated). Differential cell counts indicate a decrease in macrophages, with an increase in ciliated columnar and basal undifferentiated cells. DNA content distributions recorded 3, 5, and 7 h after exposure (see Figs. 3D, 3E, and 3F) appear similar, with peak 1

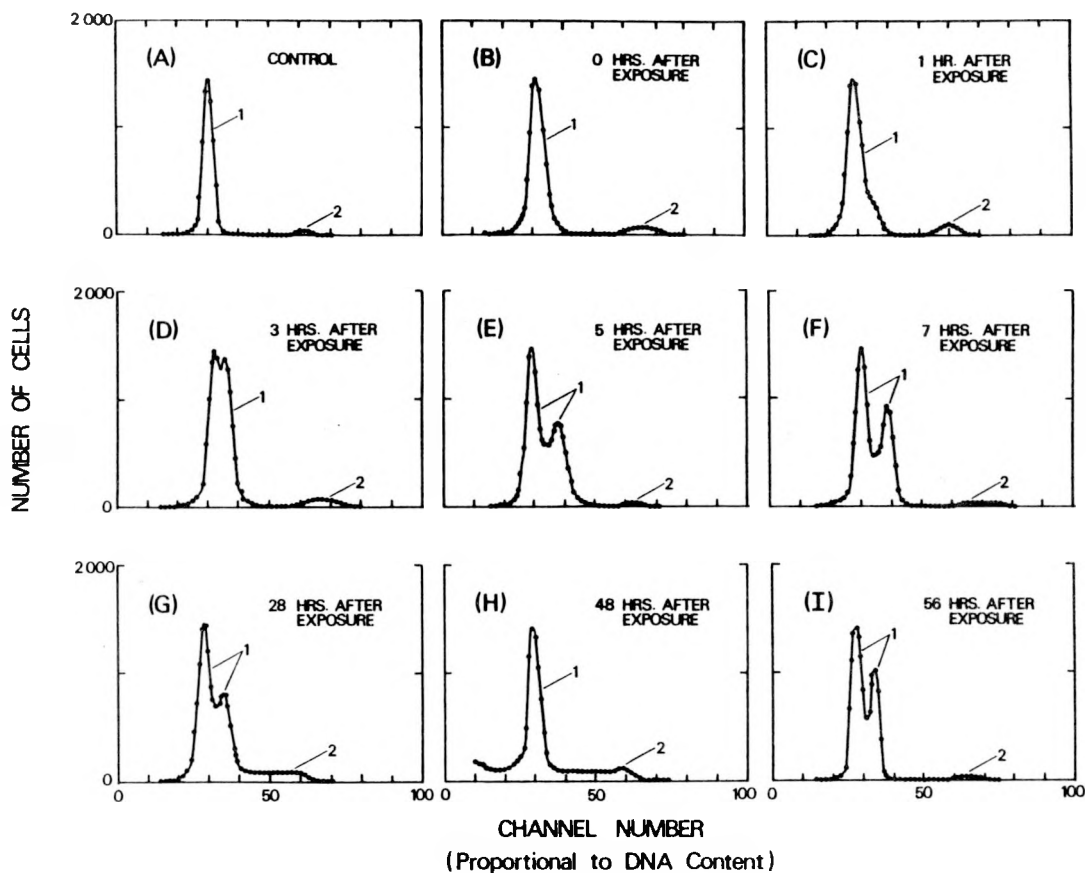


Fig. 3. DNA content frequency distribution histograms of hamster respiratory tract cells exposed by inhalation to 4 ppm of ozone for 4 h prior to sacrificing the animals at increments ranging from 0 to 56 h after exposure. Cell samples were obtained by lung lavage, fixed in 35% ethanol, stained with mithramycin, and analyzed for fluorescence.

dividing into two separate parts (bimodal distribution).

Differential cell counts continue to show a decrease in percentage of macrophages, an increase in leukocytes, and a leveling off and subsequent decrease in basal undifferentiated cells. From differential cell counts and Figs. 3D, 3E, and 3F, it can be postulated that the two separate parts of peak 1 are primarily composed of leukocytes and basal undifferentiated cells--a point that must be verified by cell separation methods. The DNA content distribution of lung cells 28 h after exposure is shown in Fig. 3G. This distribution is similar to those recorded in Figs. 3D, 3E, and 3F but has an increased percentage of cells between peaks 1 and 2. Figure 3H shows a DNA content distribution for respiratory tract cells 48 h after exposure, which appears to resemble a DNA content distribution of exponentially growing cells. Corresponding differential cell counts show primarily leukocytes

and macrophages, with a rapidly decreasing number of basal undifferentiated cells. In Fig. 3I (56 h postexposure), the DNA content distribution has reverted to resemble earlier data.

To fill in the gaps between 7 and 28, 28 and 48, 48 and 56, and beyond 56 h, hamsters were again exposed to 4 ppm ozone for 4 h and sacrificed at various times after release from exposure to complete these preliminary experiments using ozone as a known (model) toxic agent. Differential cell counts (percentages) are given in Table A-III. The data recorded in Figs. 4A through 4F closely resemble that previously recorded in Figs. 3A through 3F for hamsters 0 to 7 h after exposure. Differential cell counts were similar, with the exception that basal differential cells were present in higher percentages. The DNA content distributions at 10 and 12 h postexposure (Figs. 4G and 4H) were nearly identical, with differential cell counts indicating macrophages, leukocytes, and

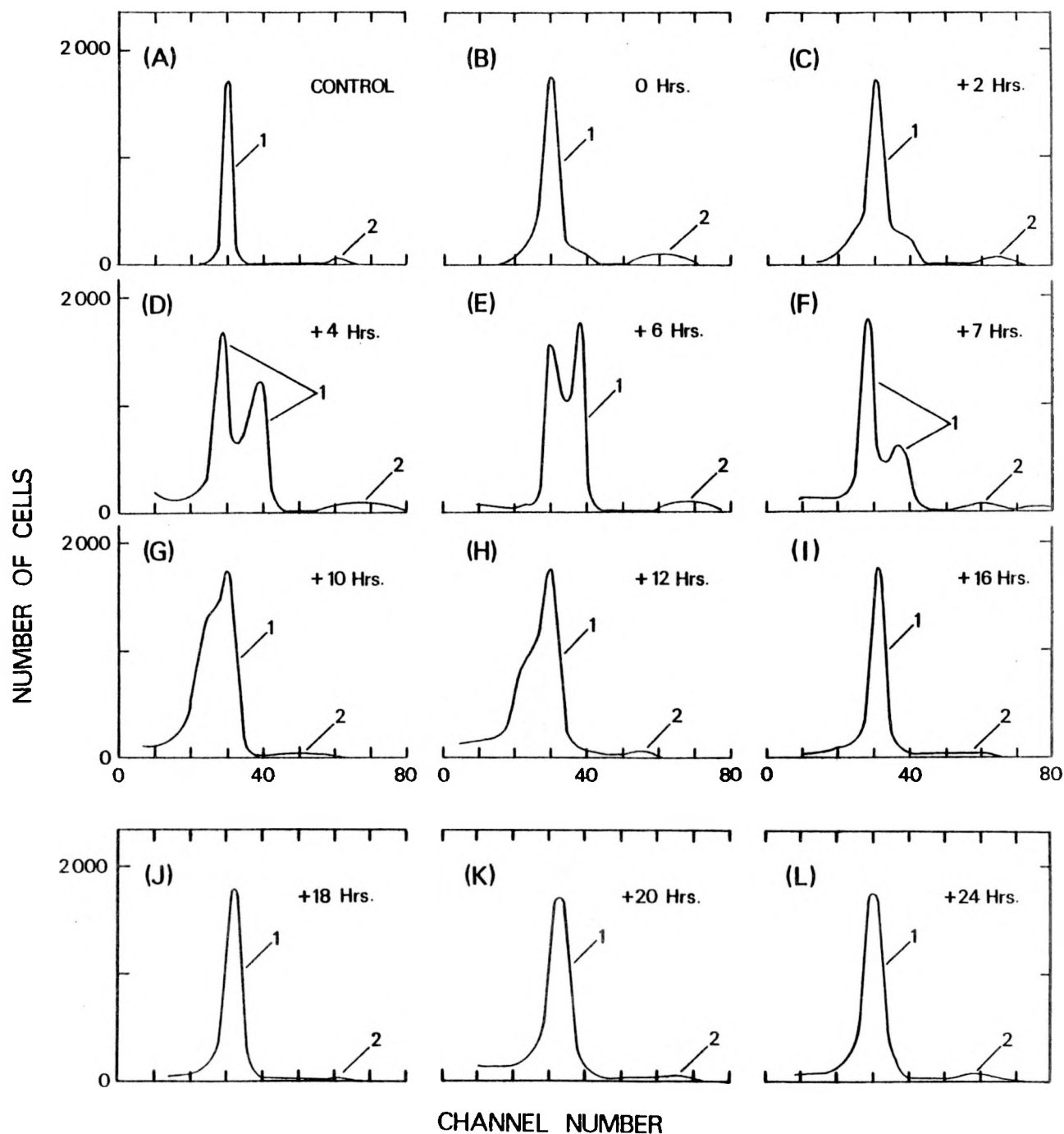


Fig. 4. DNA content frequency distribution histograms of hamster respiratory tract cells exposed by inhalation to 4 ppm of ozone for 4 h prior to sacrificing the animals at increments ranging from 0 to 72 h after exposure. Cell samples were obtained by lung lavage, fixed in 35% ethanol, stained with mithramycin, and analyzed for fluorescence.

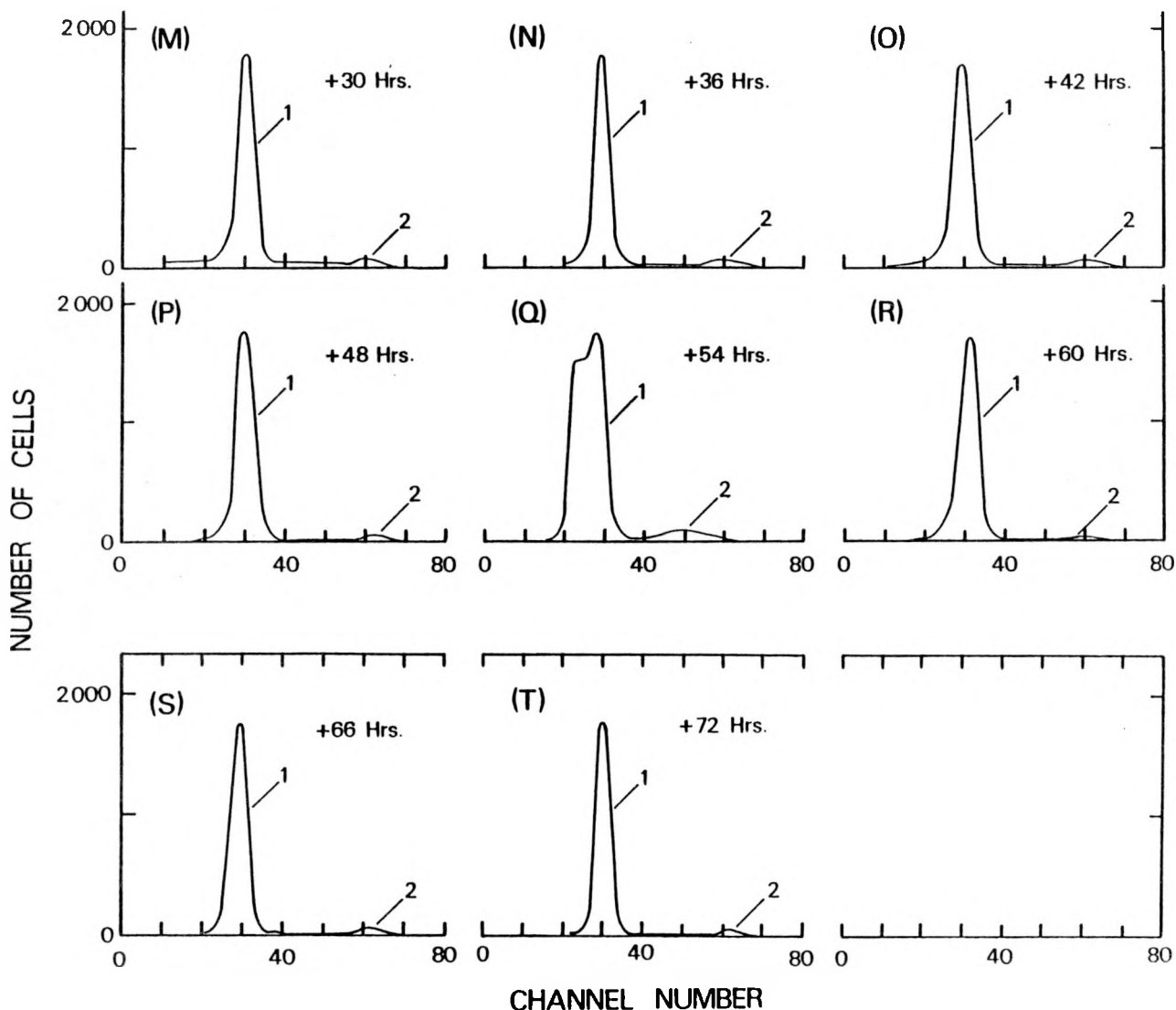


Fig. 4 (contd).

basal undifferentiated cells present. From 16 through 48 h, the DNA content distributions are similar, with some appearing normal. However, in some distributions, there was an increase in number of cells contained between peaks 1 and 2. Previous data recorded at 28 h after exposure (Fig. 3G) indicated that peak 1 consisted of two separate peaks. During this set of exposures, peak 1 remained unimodal. However, data recorded at 54 h showed a bimodal peak 1 and resembled that recorded previously at 56 h (Fig. 3I). DNA content distributions recorded after 60 h (Figs. 4R, 4S, and 4T) definitely began to appear normal, and differential cell counts stabilized. The primary

changes that occurred in the DNA content distributions as a function of exposure to ozone are (1) development of peak 1 into two peaks (bimodal); (2) an increase in binucleated cells (peak 2); and (3) an apparent increase of cells in the region between peaks 1 and 2 for some samples. There was also evidence of 3 distinct peaks developing out of peak 1 (see Fig. 5). This phenomenon occurred in only 2 samples (3 and 6 h).

To illustrate the kinetic response of cell injury to ozone, the percentage of different cells in the lavage fluid from the respiratory tract was plotted as a function of time after exposure (see Fig. 6A). The initial response was a rapid

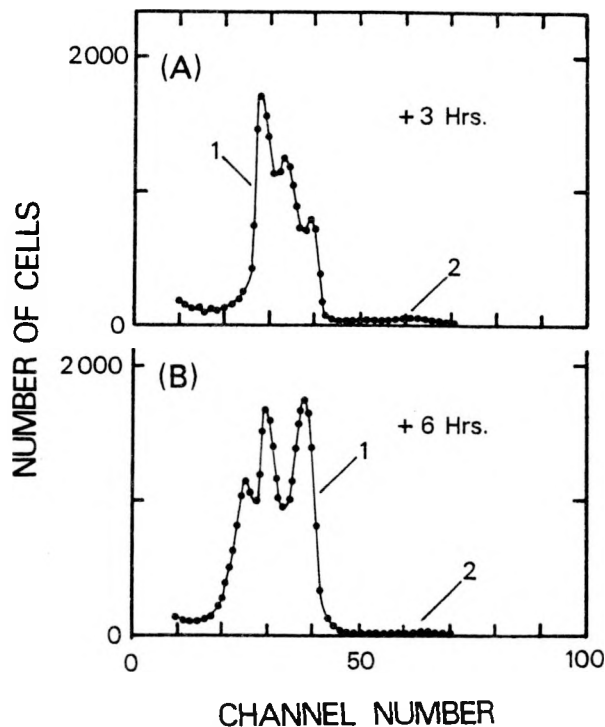


Fig. 5. DNA content frequency distribution histograms of hamster respiratory tract cells exposed by inhalation to 4 ppm ozone for 4 h prior to sacrificing the animals 3 and 6 h after exposure. Cell samples were obtained by lung lavage, fixed in 35% ethanol, stained with mithramycin, and analyzed for fluorescence.

decrease in macrophages and an increase in basal undifferentiated and ciliated columnar cells. These early effects occurred at less than 5 h after release from ozone. Leukocytes also increased but at a slower rate, reaching a maximum near 12 h. Recovery of macrophages began after 5 h, with a steady increase that reached a maximum at 13 h. After obtaining early maximum values, basal undifferentiated and ciliated columnar cells decreased slowly to normal values near 10 and 16 h, respectively. Figure 6B shows the kinetic response of macrophages and leukocytes extended to 75 h. The above results are similar to those previously reported in which rats were exposed to acute ozone levels.¹⁴

Plans are to continue using ozone as a model toxic agent for studying atypical cellular changes in relation to their measurement using flow cytometric methods. Improvements will be made in the measurement of dosage levels using better

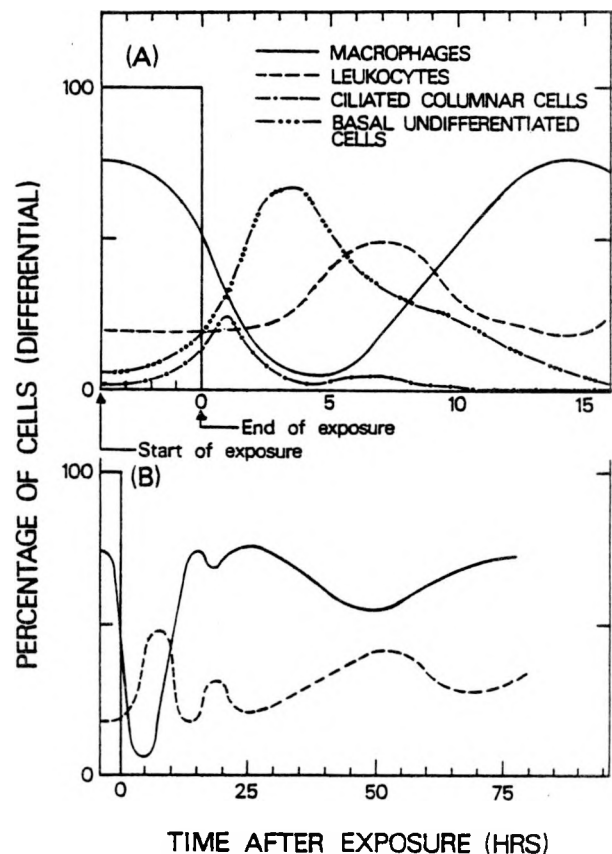


Fig. 6. Graphs of hamster respiratory tract differential cell count percentages as a function of time (h) after release from exposure to ozone (4 ppm for 4 h): (—) macrophages; (---) leukocytes; (---) ciliated columnar cells; and (-·-·-) basal undifferentiated cells. The percentages of cell types were obtained from averages of differential counts from lung lavages of animals sacrificed at increments ranging from 0 to 72 h after exposure.

instrumentation, since it was recently determined that there were inconsistencies in accurately measuring the ozone levels. To better understand what cell types correspond to the different regions within the DNA content distributions, cells will be sorted and examined cytologically. Emphasis will be placed on the correlation of cytology with instrumental measurements. This will include not only differential cell counts but determination of total cell number as a function of time after exposure. To determine if and what cell types are traversing through S-phase, autoradiographic methods will be used. Since a resolution of DNA

content measurement is important, improvements will be attempted. Also, DNA measurements will be combined with other parameters to better resolve the different cell types.

C. Flow Cytometric Quantitation of Pulmonary Macrophage Phagocytosis

Phagocytic activity, which is the primary function of pulmonary macrophages, is normally measured by exposing test animals to toxic agents, intratracheal injection of micron sized polystyrene latex particles or bacteria for a fixed time period, followed by lung lavage to remove macrophages, and microscopic enumeration of macrophages containing 1, 2, 3, etc., particles per bacteria. Described below is a new method to study qualitatively the phagocytic activity of macrophages from test animals exposed to toxic agents using fluorescent microspheres. The fluorescent excitation and emission curves for the 1.83- μm -diameter polystyrene latex particles are shown in Fig. 7. The excitation peak occurs at a wavelength of 463 nm and is a "good match" for the 457-nm violet line of the argon-ion laser. Emission is in the blue-green region of the

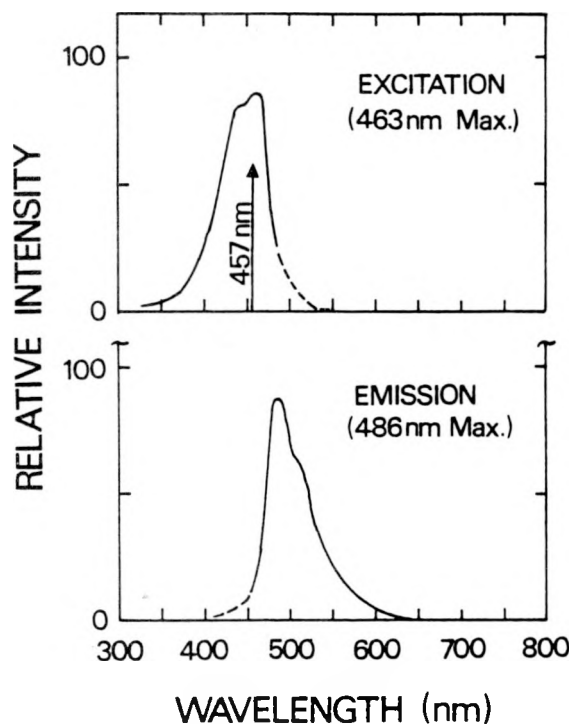


Fig. 7. Fluorescence excitation and emission spectra of 1.83- μm -diameter polystyrene latex spheres (Polysciences Corporation, Catalog No. 9847) suspended in distilled water and analyzed on an Aminco-Bowman spectrofluorometer.

spectrum. These curves were obtained from measurements on microspheres suspended in distilled water using an Aminco-Bowman spectrofluorometer.

Figure 8 shows the fluorescence distribution of 1.83- μm -diameter fluorescent spheres suspended in saline for instrument calibration. Peaks 1, 2, and 3 represent singlets, doublets, and triplets, respectively. The fluorescence and light-scatter distributions of cells and nonphagocytized spheres obtained from lavaging the respiratory tract are shown in Fig. 9. The fluorescence channel amplifier gain was identical to that recorded for the fluorescent spheres alone (Fig. 8). The fluorescence distribution (Fig. 9A) of phagocytized and nonphagocytized spheres was obtained by recording the fluorescence signals from macrophage-ingested spheres and nonphagocytized particles. Peaks 1, 2, 3, etc., represent single macrophages that contain 1 sphere or a single sphere alone; single macrophages containing 2 spheres or 2 spheres stuck together (doublet); single macrophages containing 3 spheres or 3 spheres stuck together (triplet); etc., respectively.

To distinguish between macrophages that have phagocytized spheres and nonphagocytized particles, the light-scatter method¹⁵ for cell size determination was used. Since 1.83- μm -diameter spheres are smaller than pulmonary cells, they did not appear in the cell size distribution (Fig. 9B). Peak 1, although not positively identified, is thought to be leukocytes and cellular debris. Peak 2 has been

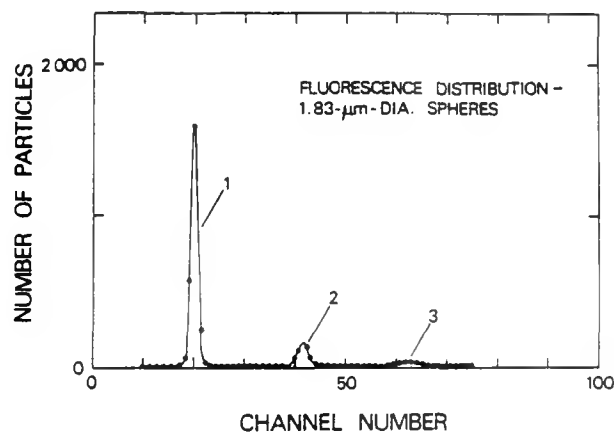


Fig. 8. Frequency distribution histogram of 1.83- μm -diameter polystyrene latex spheres (fluorescent) suspended in normal saline, excited at 457 nm using the argon laser, and analyzed for fluorescence.

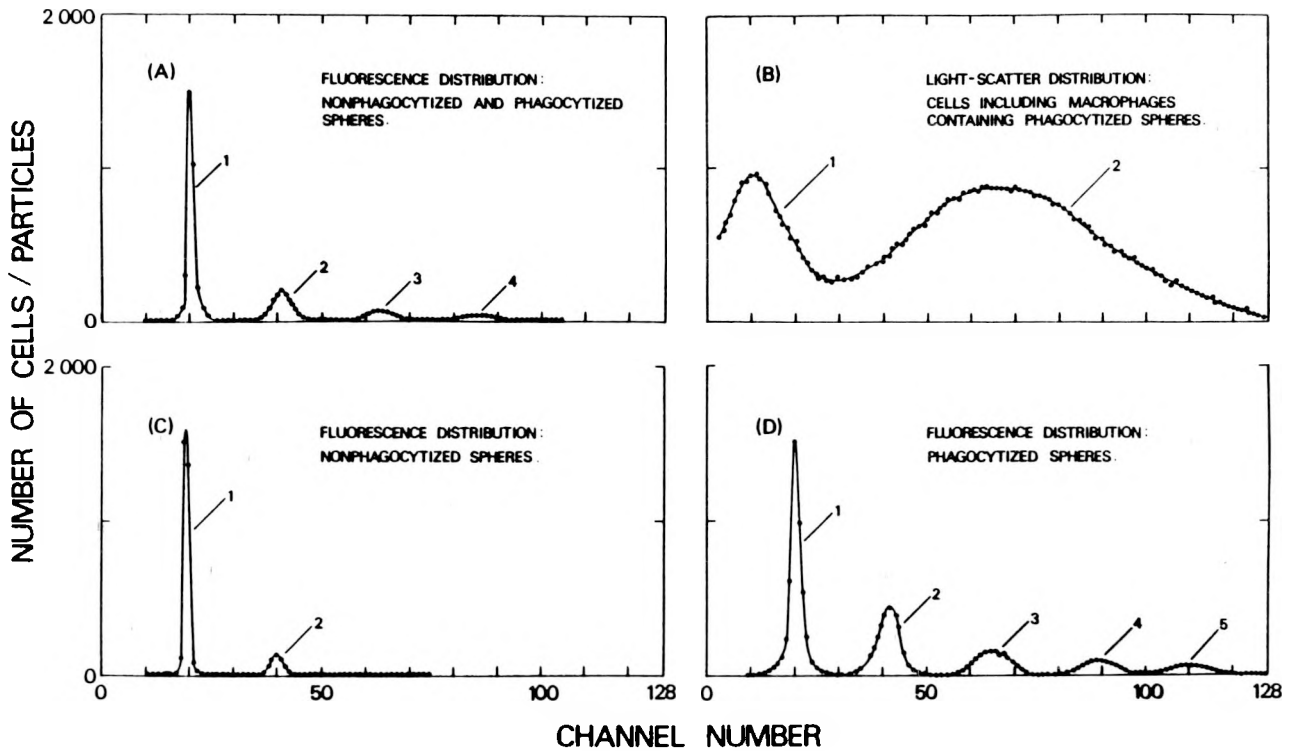


Fig. 9. Frequency distribution histograms of microspheres and cells obtained by sacrificing normal rats and lavaging the lungs with saline 2 h after instilling with 1 to 2×10^7 $1.83\text{-}\mu\text{m}$ -diameter fluorescent spheres in 0.5 ml saline: (A) fluorescence distribution of nonphagocytized and phagocytized spheres obtained by recording all fluorescence signals; (B) light-scatter distribution (size) of cells, including macrophages containing phagocytized spheres; (C) fluorescence distribution of nonphagocytized spheres obtained by recording only those fluorescence signals not associated with light-scatter signals; and (D) fluorescence distribution of phagocytized spheres obtained by recording only those fluorescence signals associated with light-scatter signals. Cells were fixed in 35% ethanol, excited at 457 nm using the argon laser, and analyzed for fluorescence.

identified by sorting and microscopic examination and represents macrophages that do or do not contain phagocytized spheres. Therefore, by requiring fluorescence signals to be or not to be in coincidence with light-scatter signals (cells), nonphagocytized spheres and macrophages that have phagocytized spheres can be separated. For example, Fig. 9C shows the fluorescence distribution of nonphagocytized spheres obtained by displaying only those fluorescence signals having no light-scatter signal associated with them. Peaks 1 and 2 represent singlets and doublets (spheres). The fluorescence distribution (Fig. 9D) of macrophages having phagocytized spheres similarly was obtained by displaying only the fluorescence signals from cells that also scattered light (see Fig. 9B). Cells contained within peaks 1 to 5 (Fig. 9D) represent macrophages that have phagocytized 1 to

5 spheres, respectively, as identified by sorting cells from each peak (Fig. 10).

Since cells contained in peak 2 of the light-scatter distribution (Fig. 9B) are primarily macrophages, including those that have and have not phagocytized spheres, they can be combined with those that have phagocytized spheres to determine quantitatively the phagocytic activity. This can be accomplished by simultaneously displaying the distribution of fluorescence signals (Fig. 9D) associated with phagocytized spheres and the light-scatter distribution of total cells, including macrophages containing spheres (Fig. 9B). The percentage of macrophages having phagocytized spheres (phagocytic activity) then can be determined by dividing the number of cells in each peak (Fig. 9D) by the total macrophages (peak 2, see Fig. 9B).

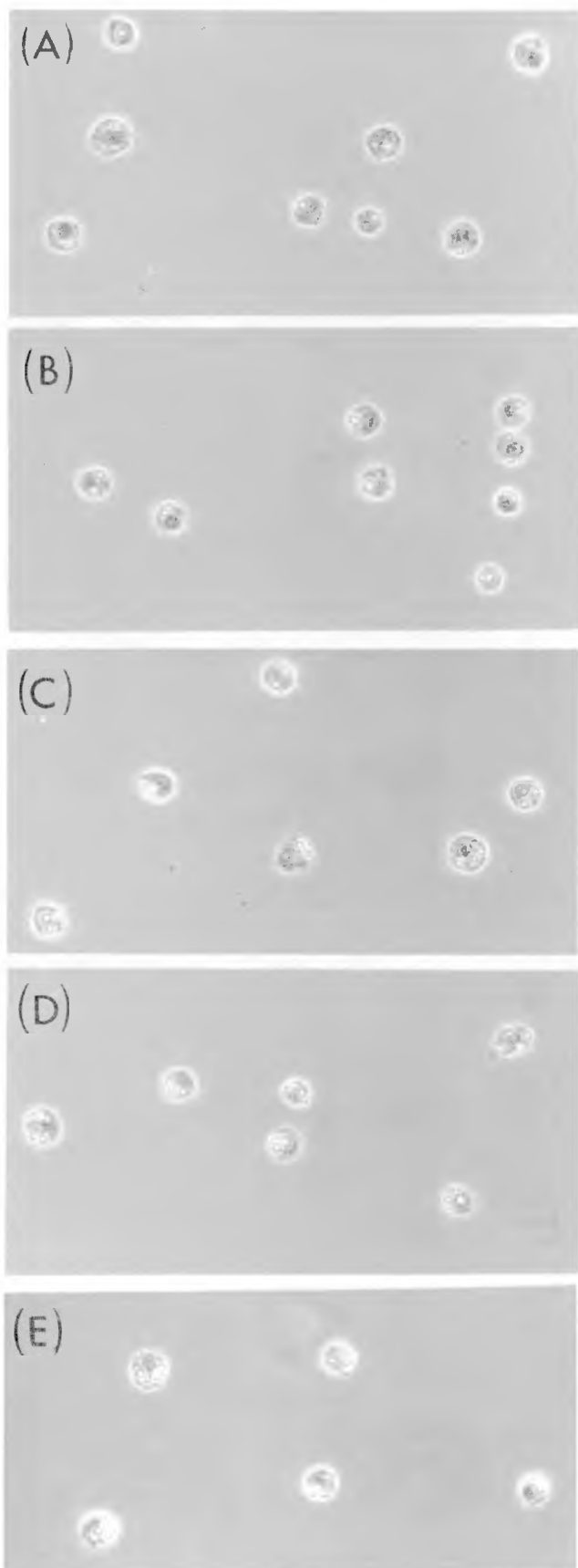


Fig. 10. Photomicrographs of rat macrophages containing 1.83- μ m-diameter microspheres separated from peaks 1 through 5 of the fluorescence distribution (Fig. 9): (A) cells separated from peak 1 containing 1 microsphere per cell; (B) cells separated from peak 2 containing 2 microspheres per cell; (C) cells separated from peak 3 containing 3 microspheres per cell; (D) cells separated from peak 4 containing 4 microspheres per cell; and (E) cells separated from peak 5 containing 5 microspheres per cell ($\times 490$).

To demonstrate that peak 2 (Fig. 9B) contains both macrophages that have and have not phagocytized spheres, respective light-scatter distributions were recorded for illustrative purposes. Figure 11A shows the light-scatter distribution (size) of cells containing phagocytized spheres obtained by displaying only those light-scatter signals associated with fluorescence. Cells

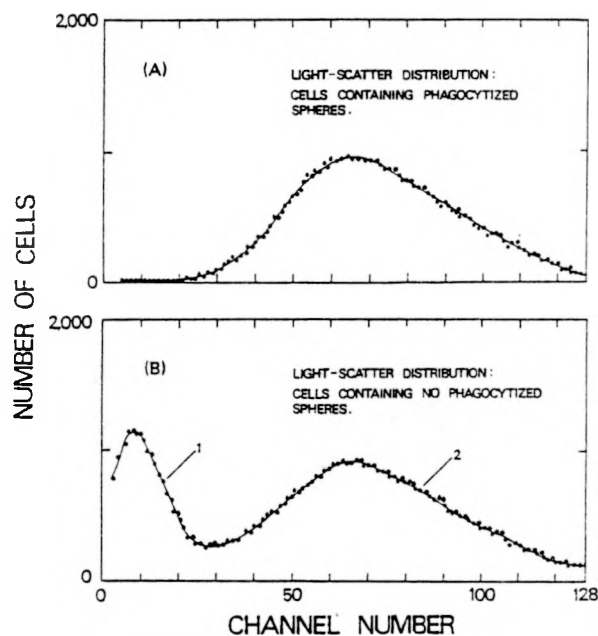


Fig. 11. Light-scatter (size) frequency distribution histograms of cells obtained by sacrificing normal rats and lavaging the lungs with saline 2 h after instilling with 1 to 2×10^7 1.83- μ m-diameter fluorescent spheres in 0.5 ml saline: (A) size distribution of cells (macrophages) containing phagocytized spheres obtained by recording only those light-scatter signals associated with fluorescent signals; and (B) size distribution of cells containing no phagocytized spheres obtained by recording only those light-scatter signals not associated with fluorescence signals. Cells were fixed in 35% ethanol prior to analysis.

contained within this distribution were separated and identified as only macrophages having phagocytized 1, 2, 3, etc., microspheres. The light-scatter distribution of cells containing no spheres (see Fig. 11B) similarly was obtained by displaying only those cell size signals not associated with fluorescence. Peak 1 represents leukocytes and peak 2 macrophages that have not phagocytized spheres.

A new method for quantitative measurement of phagocytic activity of pulmonary macrophages *in vivo* using advanced flow analysis methods has been described. This technique has potential for permitting rapid and accurate determination of phagocytosis and will be subsequently used to assay for cytotoxicity. Tests are under way to determine if phagocytic activity can be combined with the measurement of other biochemical parameters and to evaluate further the methodology described above.

D. Evaluation of New Methods for Measuring DNA, Protein, and Alkaline Phosphatase

Efforts are continuing in the development of new methods and in the improvement of existing ones for measuring DNA content, protein, enzymes, and other biochemical parameters. This is an important area of the program, since accuracy in flow

cytometry and subsequent measurement of cytological and biochemical indicators of damage depend upon quantitative staining of cellular constituents.³ Staining methods are normally evaluated first using model cell systems (cultured), prior to analyzing heterogeneous populations (e.g., blood, cervical, respiratory, and dermal cells), which often have different staining characteristics. For example, the propidium iodide-fluorescein isothiocyanate (PI-FITC) method for measuring DNA and protein gives excellent results using a variety of cultured cell lines.^{11,16} However, when respiratory tract cells were analyzed using PI-FITC, the red fluorescence PI (DNA content) distribution was consistently broad and sometimes skewed.⁵ Similar but improved results have been obtained using mithramycin and rhodamine 640 for measuring DNA content and protein, respectively.⁹ This method, which requires dual-laser excitation, is presently being evaluated. Protein distributions appear similar regardless of the method used. Bis-[4-aminophenyl]-1,3,4-oxadiazol (BAO) has been recently combined with PI for analyzing protein and DNA content in cultured cell lines,¹² as illustrated in Fig. 12. These distributions are similar to that reported using PI-FITC and mithramycin-rhodamine 640.

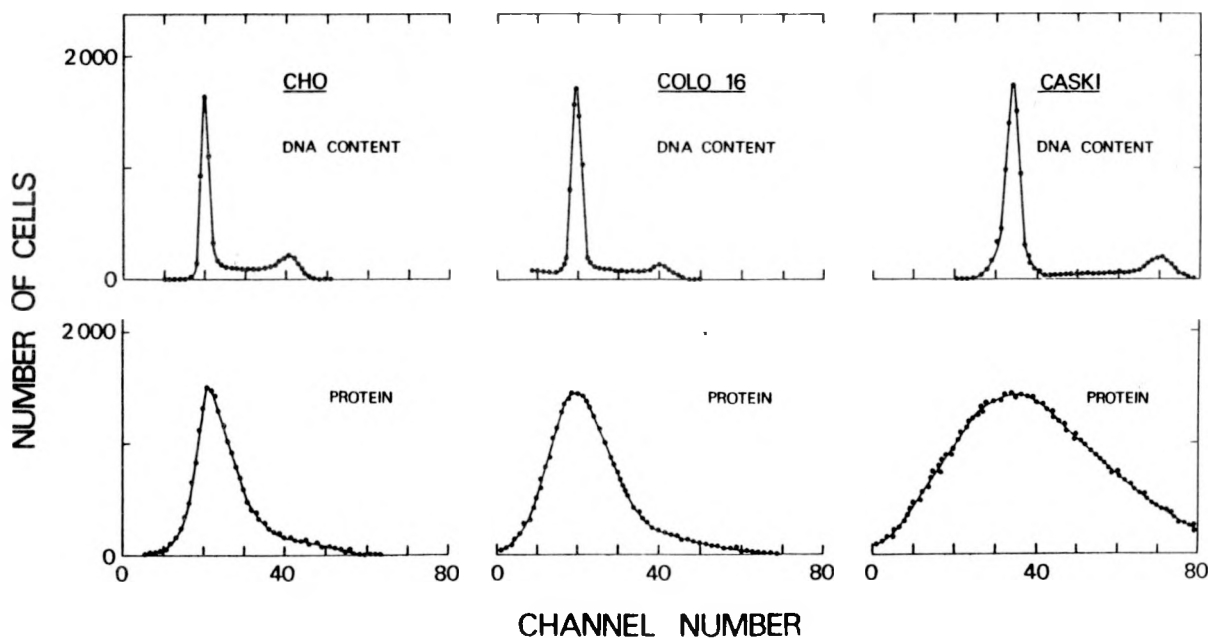


Fig. 12. DNA content and total protein frequency distribution histograms of lines CHO, COLO 16, and CaSKI cells fixed in 70% ethanol, stained with propidium iodide (DNA) and BAO (protein) excited with the uv argon laser lines, and analyzed for red and green fluorescence. Both DNA content and protein fluorescence amplifier measurement channels (gains) were fixed.

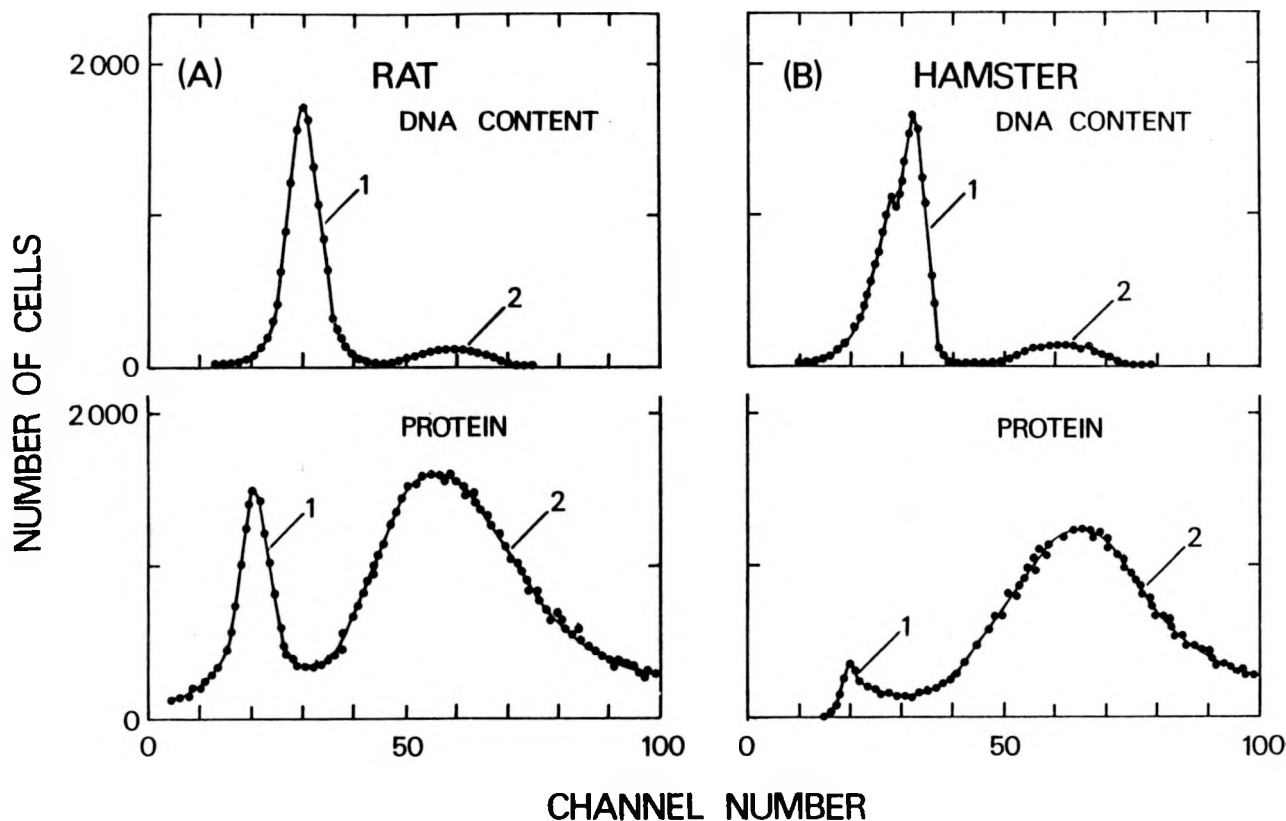
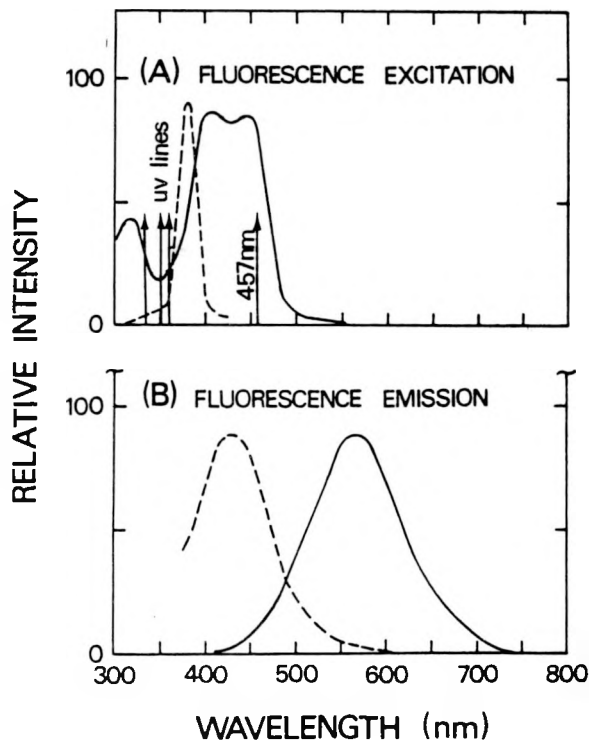


Fig. 13. DNA content and total protein frequency distribution histograms of normal rat and hamster respiratory tract cells obtained by lavaging the lungs with saline, fixing in 35% ethanol, and staining with propidium iodide (DNA) and BAO (protein), excited with the uv argon laser lines, and analyzed for red and green fluorescence.



Respiratory tract cells also have been analyzed using the PI-BAO method (Fig. 13). The DNA content distributions are broad and slightly skewed. Peaks 1 and 2 represent mononucleated (2C DNA content) and binucleated cell doublets (4C DNA content), respectively. The protein distributions are similar to that previously reported.^{5,9} Peaks 1 and 2 most likely represent leukocytes and macrophages/basal undifferentiated/ciliated columnar cells, respectively.

BAO also can be combined with mithramycin using dual-laser excitation. The fluorescence excitation and emission curves for BAO and mithramycin bound to protein and DNA are shown in Fig. 14.

Fig. 14. Excitation and emission spectra for mithramycin (—) bound to DNA and BAO (---) bound to bovine serum albumin obtained on an Aminco-Bowman spectrofluorometer. The uv and 457-nm dual-laser excitation lines are shown for illustrative purposes.

BAO and mithramycin are excited using the uv and 457-nm lines from the krypton and argon lasers, respectively. The fluorescence emission is partly overlapping. Figure 15 shows the DNA content and protein distributions measured on line CHO cells stained with mithramycin and BAO and analyzed using dual-laser excitation methods.¹² Experiments are planned to stain and analyze animal respiratory tract cells using this technique.

To initiate development of a method to quantitate alkaline phosphatase activity in lung cells, preliminary experiments have involved using naphthol AS-BI as a fluorogenic substrate.¹⁷ Normal respiratory tract cells from hamsters and rats were reacted with naphthol AS-BI and excited in the uv, and the resulting fluorescence distributions shown in Fig. 16 were obtained. Both distributions contain multiple peaks whose corresponding cells have not been identified. Peaks 1 and 2 of Fig. 16A most likely correspond to leukocytes and macrophages, respectively. Further experiments are planned to characterize normal respiratory tract

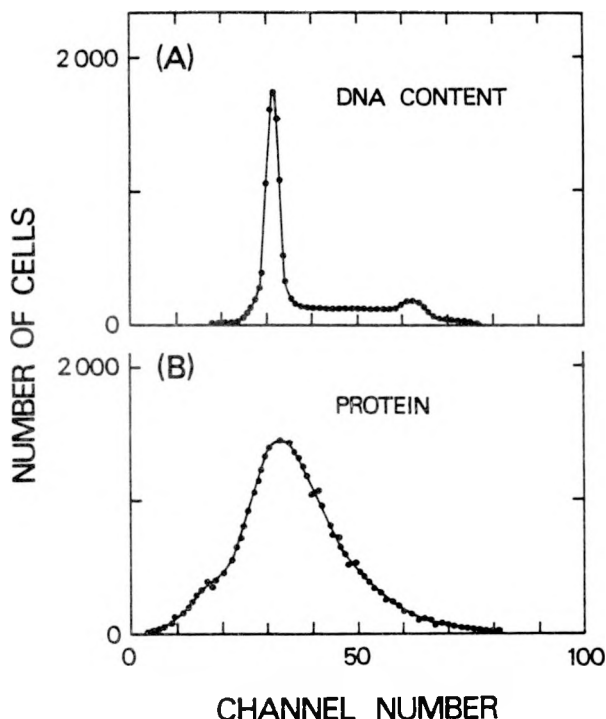


Fig. 15. DNA content and total protein frequency distribution histograms of line CHO cells fixed in 70% ethanol, stained with mithramycin (DNA) and BAO (protein), excited in the uv and at 457-nm using dual-laser methods, and analyzed for two-color fluorescence properties.

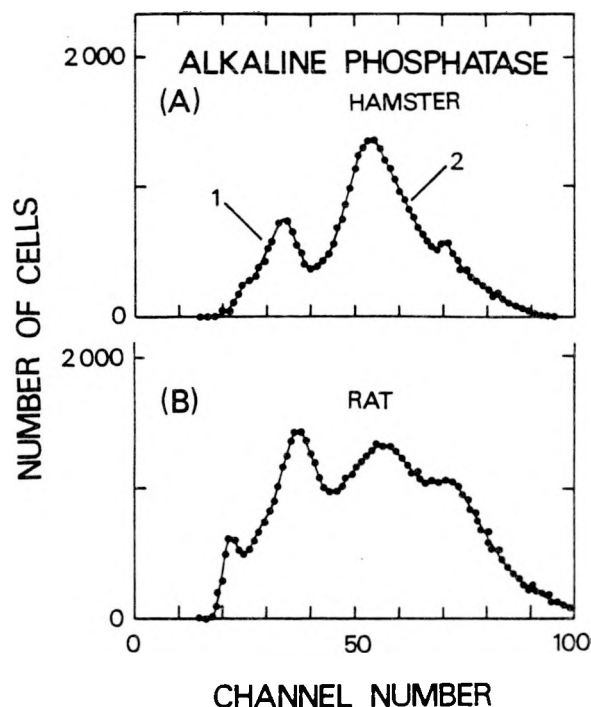


Fig. 16. Frequency distribution histograms of the relative alkaline phosphatase level in cells obtained from a normal hamster and rat by lavaging the lungs with saline, fixing in 1.5% glutaraldehyde, reacting with naphthol AS-BI, and analyzing for fluorescence. The horizontal scale (channel number) is proportional to approximately 3 log decades.

cells based on alkaline phosphatase activity prior to exposing the animals.

Plans are to continue development and evaluation of methods for measuring DNA-protein and cellular enzymes. Emphasis will be placed on finding a two-color fluorescence staining technique for determining DNA and protein, with improved resolution of peak 1 of the DNA distribution (2C DNA content cells), prior to measuring these two parameters on exposed animals. Methods for measuring differences in enzyme levels also will be emphasized. Preliminary characterization studies will be completed on the naphthol AS-BI method for detecting alkaline phosphatase levels in cells. Experiments are also planned to use a modification of this technique to measure acid phosphatase. Other fluorogenic substrates will be evaluated, when possible, for potential measurement of enzymes present in respiratory tract cells.

IV. FUTURE PLANS

Experimental goals during the next 6 months are to (1) repeat and extend DNA content measurements from hamsters exposed to particulates of oil shale and silica via intratracheal injection, to include examining lung-cell samples at other times after exposure, improve and correlate cytology, including total cell number counts with DNA measurements, and begin to examine respiratory tract cells from hamsters exposed in inhalation chambers; (2) improve the present experimental setup for exposing animals to gaseous agents such as ozone, repeat preliminary experiments of exposing animals to an acute level of ozone, and improve and correlate cytology with DNA measurements; (3) continue the development of using fluorescent microspheres to measure phagocytic activity, with emphasis on exposing animals to known agents that cause an increase or decrease in phagocytosis, computerizing data analysis, and combining phagocytic activity with other cellular parameters; (4) improve the resolution of DNA-protein measurements in respiratory tract cells; and (5) continue the development of enzyme staining methods for determining alkaline and acid phosphatase activity.

V. ACKNOWLEDGMENTS

The authors thank J. L. Horne (assistance in instrumentation development and illustrations); J. Grilly (photography), L. M. Holland (consultations concerning animal exposures and toxicology), E. M. Sullivan (manuscript preparation), and D. L. Coffin of the United States Environmental Protection Agency, Research Triangle Park, North Carolina (assistance in developing a method for measuring macrophage phagocytic activity).

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TABLE A-I
COMPARISON OF WAVELENGTH AND POWER AVAILABILITY WITH HIGH-POWER ARGON AND KRYPTON LASERS

	Wavelength (nm)	Laser Power (W)	
		Argon (CR-10)	Krypton (3000K)
<u>Ultraviolet</u>	333, 351, 363	1.0	-
	337, 350, 356	-	2.0
<u>Violet</u>	406	-	0.9
	413	-	1.5
	415	-	0.1
	457	0.6	-
<u>Blue</u>	465	0.2	-
	468	-	0.5
	472	0.4	-
	476	1.5	0.4
	482	-	0.4
	488	3.5	-
	496	1.2	-
<u>Green</u>	501	0.75	-
	514	4.5	-
	520	-	0.7
<u>Yellow</u>	530	-	1.5
	568	-	1.1
<u>Red</u>	647	-	3.5
	676	-	0.9
<u>Infrared</u>	752	-	1.2
	793, 799	-	0.3

TABLE A-II
DIFFERENTIAL CELL COUNTS OF RESPIRATORY TRACT SAMPLES FROM HAMSTERS EXPOSED TO 4 PPM OZONE FOR 4 h^a

Hamster Number	Time after Exposure (h)	Macrophages (%)	Leukocytes (%)	Basal Undifferentiated Cells (%)	Ciliated Columnar Cells (%)
A	Control	74	18	7	1
B	0	53	18	17	12
C	1	30	20	27	23
D	3	10	23	63	4
E	5	5	40	52	3
F	7	18	47	33	2
G	28	41	53	6	0
H	48	40	58	2	0
I	56	24	75	1	0

^aDifferential counts were determined microscopically from samples obtained by lavaging the lungs t h after exposure.

TABLE A-III

DIFFERENTIAL CELL COUNTS OF RESPIRATORY TRACT SAMPLES FROM HAMSTERS EXPOSED TO 4 PPM OZONE FOR 4 H^a

Hamster Number	Time after Exposure (h)	Macrophages (%)	Leukocytes (%)	Basal Undifferentiated Cells (%)	Ciliated Columnar Cells (%)
A	Control	76	16	6	2
B	0	71	13	4	12
C	2	20	29	22	29
D	4	5	33	62	0
E	6	10	46	39	5
F	7	18	47	33	2
G	10	49	23	26	0
H	12	63	25	15	0
I	16	72	27	1	0
J	18	65	35	0	0
K	20	70	23	7	0
L	24	74	23	3	0
M	30	72	22	6	0
N	36	65	30	5	0
O	42	58	34	8	0
P	48	54	43	3	0
Q	54	60	38	2	0
R	60	61	37	2	0
S	66	73	23	4	0
T	72	66	32	2	0

^aDifferential counts were determined microscopically from samples obtained by lavaging the lungs t h after exposure.

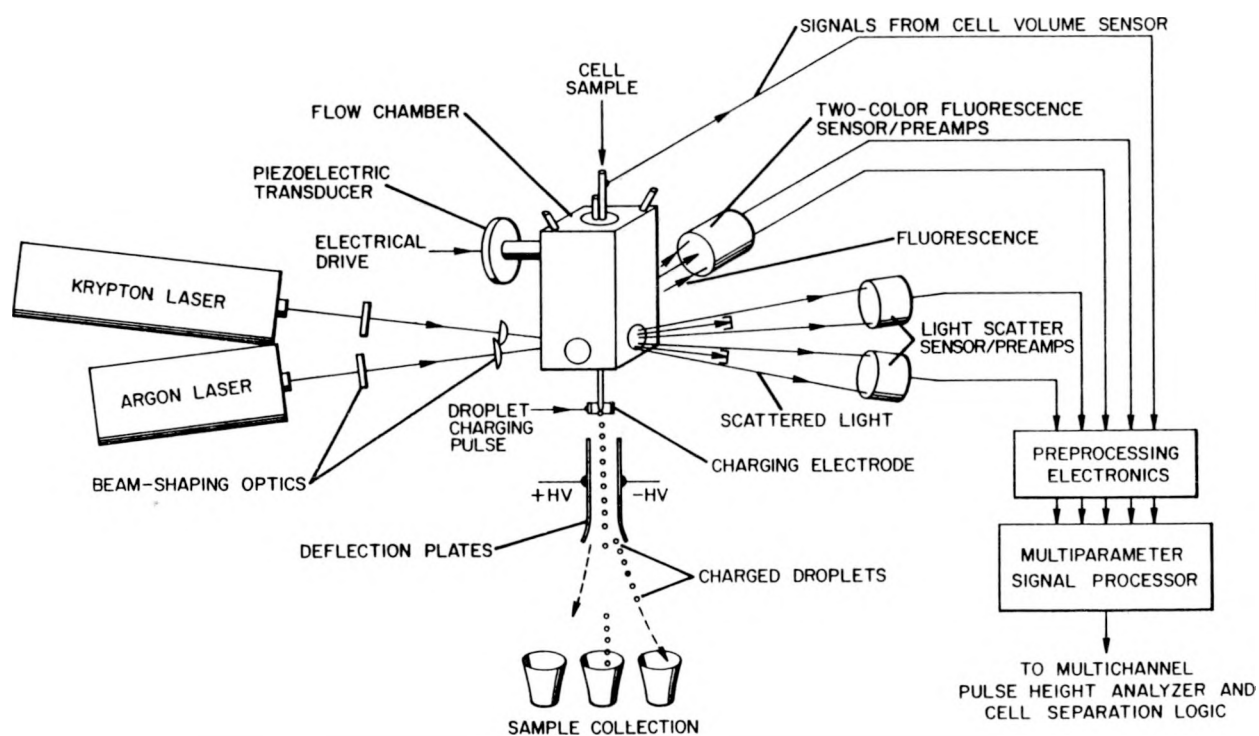


Fig. A-1. Multiparameter cell analysis and sorting instrument.