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TITLE: FLOW CYTOMETRY FOR HEALTH MONITORING IN SPACE

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**FLOW CYTOMETRY FOR HEALTH MONITORING IN SPACE**

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**Short Title: Jett, Martin, Saunders and Stewart: Flow Cytometry**

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## **ABSTRACT**

**Monitoring the health of space station or lunar base residents will be necessary to provide knowledge of the physiological status of astronauts. Flow cytometric techniques are uniquely capable of providing cellular, chromosome, hormone level and enzyme level information. The use of dyes provides the basis for fluorescently labeling specific cellular components. Laser induced fluorescence from stained cells is quantitated in a flow cytometer to measure cellular components such as DNA, RNA and protein. One major application of a flow cytometer will be to perform a complete blood count including hematocrit, hemoglobin content, and numbers of platelets, erythrocytes, granulocytes, lymphocytes and monocytes. A newly developed flow cytometry based fluoroimmunoassay will be able to measure levels of serum enzymes and hormones. It will also be possible to quantitate radiation exposure and some forms of chromosome damage with flow cytometric measurements. With relatively simple modifications to existing technology, it will be possible to construct a flight rated cytometer.**

## Introduction

The health maintenance facility of the space station, moon base or any extended voyage to Mars or beyond must be capable of routine hematological, immunological and blood chemistry measurements. Even with an expert in these areas available on these missions, routine measurements and assays would require several conventional clinical instruments. Flow cytometry, a technology under development for the past 20 years (Steinkamp, 1984; Braylan, 1983; Melamed, 1979), is uniquely suited to perform the types of measurements required in a health maintenance facility. In addition, the capabilities of the flow system described below will be a valuable source of information in space adaptation research. During the development of flow cytometry, Los Alamos has been at the forefront and is a recognized leader in instrumentation advances as well as the biological applications of the technology.

The ability of a flow cytometer to make rapid measurements on cells and subcellular components requires the localization of the particles to a small probe volume ( $10^{-12}$  liter) and rapid transport of the particles through that probe volume. By employing the principal of hydrodynamic focusing (Crossland-Taylor, 1953) depicted in Figure 1, the cells in the flowing sample

stream are confined to a central core on the order of 10 micrometers in diameter as the cells pass through a focused laser beam. The laser light interacts with the cell in a number of ways. Measurement of the light scattered by a cell gives information about cell size in the forward direction and about cell shape and surface morphology when measured at 90 degrees to the laser beam propagation direction. In some systems, it is also possible to determine cell size by electrical resistance measurements via the Coulter principle.

The laser beam will also excite fluorescent dyes used to label specific cellular constituents. Photo detectors measure the amount of fluorescence emitted by the dye molecules as a cell passes through the laser beam, providing a quantitative measurement of the stained cellular component. Table 1 lists many of the cellular constituents which have been quantitated by fluorescence intensity measurements.

Other types of measurements which have been made with flow cytometers are listed in Table 2. Fluorescence polarization measurements provide information about the rigidity of the local environment of the fluorescing molecule. This technique has been used to quantify membrane fluidity. The time parameter correlates any flow measurement with time to

provide a history of a changing parameter such as a fluorescence. These kinetic measurements can be used to study the turn over rate of fluorogenic substrates to give a measure of enzyme activity or can be used for dye binding studies.

Flow cytometers have proven to be an important tool in a number of fields of biomedicine. Blood cell volume distributions, one of the first measurements made with a flow system, are now routinely made in most hospitals. The first fluorescence measurements quantitated the amount of DNA per cell by measuring the amount of fluorescence emitted by a dye stoichiometrically bound to the cellular DNA as the cell passed through the laser beam (Van Dilla, 1959). Figure 2 is a typical DNA histogram for an exponentially growing cell population. The distribution, which contains data from approximately 75,000 cells, was obtained in less than two minutes. In specially designed flow systems, cells and chromosomes can now be analyzed at rates up to 20,000 per second.

#### Measurements Relevant to Space Medicine

There are several classes of flow cytometric measurements which provide information important in health maintenance. They include routine hematological assays, immunological assays including immune cellular

function as well as determination of serum enzyme and hormone levels, quantitation of radiation exposure and quantitation of chromosome damage.

### Hematological Measurements

One major application of a flow cytometer is the complete blood count using only microliters of blood. Such a blood count would include the hematocrit, hemoglobin content, as well as the numbers of platelets, erythrocytes, granulocytes, lymphocytes and monocytes.

**Red Blood Cells:** The number of red blood cells per cubic millimeter, their volume and hematocrit distribution can be determined in a flow system. The number density is measured by adding a small volume of plastic microspheres at a known density to the sample. The size of the microspheres is selected such they do not interfere with the blood cell volume measurements. By counting both the number of microspheres and the number of cells, the volume of the sample analyzed is determined from which the density of cells can be calculated. With an electronic cell volume measurement station in the flow cytometer, the volume distribution of the red blood cells can be measured. From this information the hematocrit of the erythrocytes can be determined. Since hemoglobin absorbs strongly at 420 nm, it will also be possible to

measure the hemoglobin content on a per cell basis.

**White Blood Cells:** In a similar manner, the number of white blood cells per cubic millimeter can be determined. As shown in Figure 3, forward and 90 degrees light scattering measurements can be used to subdivide the white blood cells into granulocytes, lymphocytes, and monocytes. Using fluorescently labeled monoclonal antibodies (Hoffman, 1980) specific for these subsets of leukocytes, it will also be possible to apply and refine currently available methodologies to resolve the granulocyte subsets: neutrophils, eosinophils, and basophils and the lymphocyte subsets: B cells, T helper cells, and total T cells (as shown in Figure 4). The methods can be made simple and packaged in the form of a prepared kit to which a drop of blood from the finger would be added and then analyzed. After measurement, the data would be either analyzed on board or transmitted to earth. Multiparameter analysis of the acquired data will resolve each cell population found in the peripheral blood from the others.

#### **Functional Activity of Leukocytes**

The leukocyte measurements cited above provide information about the immunological status of an individual. In addition to the just enumerating the different cell types, functional assays can also be performed. For example, the

phagocytic activity of monocytes and granulocytes can be measured by incubating the cells with small (1-2 micrometer) fluorescent microspheres for a short time and then analyzing the sample in a flow system to determine the amount of microsphere fluorescence which is associated with each cell (Steinkamp, 1982). It is also possible to determine the number of particles up to approximately 20 ingested by each cell. There are a number of techniques for determining cell viability with fluorescent dyes. Thus cytotoxicity assays can be performed with a flow system (Horan, 1977).

In addition to phagocytosis and cell viability, it is also possible to assess the microbicidal activity of granulocytes and monocytes. The major biochemical pathway for bacterial killing by these leukocytes is the hexose monophosphate shunt which generates superoxide anion and hydrogen peroxide. The activity of the shunt can be quickly assessed on a cell-by-cell basis by measuring the autofluorescence above 400nm after excitation at 350nm. Thus, both phagocytosis, the major ingestive pathway and microbicidal activity can be quickly measured using a flow cytometer.

#### Serum Hormone and Enzyme Levels

The levels of a variety of hormones and enzymes in blood serum are indicators of a number of aspects of an individual's state of health. For

example, elevated levels of creatine kinase are indicative of a recent heart attack.

A method of measuring, with a flow cytometer, the concentration of serum proteins and enzymes of importance has been developed (Saunders, 1985). The basis for this new assay is similar to a competitive binding radioimmunoassay except that small fluorescent microspheres are displaced instead of radioactively labeled molecules. The assay consists of coating large (10 micrometer diameter) nonfluorescent microspheres with antibodies to the molecule of interest. These large antibody coated spheres are then incubated in the liquid sample being assayed. The free antigen molecules in the sample bind to the large spheres via the antibody-antigen interaction. The number of free antibody sites on the large spheres is inversely proportional to the amount of free antigen in the sample. At this point, small (0.1 micrometer diameter) fluorescent antigen coated microspheres are added to the sample for another incubation period. These small fluorescent spheres bind to the remaining free antibody sites on the large spheres (Figure 5).

Without separation of bound from free fluorescent spheres, the whole sample is analyzed using a flow cytometer. There is a very small DC fluorescence level present due to the free small spheres that is ignored

electronically. The data acquisition system is set to record the fluorescence only when a 10 micrometer sphere is detected by light scatter. As the antigen concentration increases and binds more antibody sites on the large sphere, fewer small fluorescent microspheres can bind and there is less fluorescence associated with the large spheres. This loss of fluorescence is proportional to the antigen concentration (Figure 6).

With this assay, a detection limit of  $10^{-12}$  molar has been achieved for horse radish peroxidase. In a sandwich assay based on similar principles, a detection limit of  $10^{-14}$  molar has been reached for the same antigen. In theory, this type of assay could be developed for any molecule which is immunogenic.

One of the advantages of this type of assay is that it is homogeneous alleviating the necessity of separating bound from free label chemically - a procedure which both takes time and also results in decreased precision because the equilibrium is disturbed. Further advantages include the fact that no radiolabeled compounds are used and the sensitivity of the system is better than many radioimmunoassays.

#### Determination of Radiation Exposure

It has been recently shown (Nusse, 1984) using tissue culture system

that the dose of radiation received by cells can be determined by a flow cytometric assay. The assay consists of determining the number of micronuclei formed in the cultured cells after irradiation. The cell suspension is treated with a detergent, stained with a DNA specific dye, and analyzed on a flow cytometer. The micronuclei appear in the distribution as objects with low DNA content. By plotting the ratio of the number of micronuclei detected to the total number of cells analyzed as a function of dose, a dose response curve can be generated. A flow cytometer with reasonable sensitivity is needed since the DNA content of the micronuclei is on the order of that of the smallest human chromosomes which is approximately 1/100th of the human genome.

#### Chromosome Damage

A relatively recent development in flow cytometry is flow karyotype analysis (Carrano, 1979). The measurement basis for a flow karyotype is quantitation of chromosome DNA content. Some techniques use two lasers to excite two dyes with different base pair specificities. Present day techniques resolve the human karyotype into 21 groups with relative ease based on two color fluorescence measurements. Since, at the present time, chromosome banding patterns can not be resolved by flow measurements, only DNA content

per chromosome can be measured. Thus, not all types of subtle chromosome damage can be detected by flow cytometric measurements. However, gross deletions, breaks, and insertions can be detected.

#### System considerations for the Space Environment

There are no perceivable incompatibilities between zero or low gravity environments and flow technology. However, some design changes will be necessary. Since lasers are bulky, very inefficient at producing light and have high power requirements, another illumination source will be necessary. Several flow systems have been built with mercury arc lamps for light sources. Another possibility is to use the sun as an illumination source. Air pressurized fluid tanks will have to be replaced with pulsation free fluid pumps to avoid problems in a microgravity environment. Analog and digital electronic components can be miniaturized to meet requirements of size, power consumption and cooling. All data recorded is in digital form and is thus compatible with data links for transmission back to earth. A system can be designed with automated alignment procedures controlled by a preprogrammed microprocessor. With additional development, it will be possible to record images of cells as they pass through the light beam. Images recorded in this manner can be analyzed for morphological features to determine cell types,

abnormal cytology and other parameters of interest. In addition to the instrumentation development, sample preparation protocols will have to be modified and/or developed with appropriate packaging of reagents for prolonged flights. We do not envision any difficulty in the development of one or two step kits for any of the desired analytical endpoints.

#### Conclusion

A space flow cytometer will provide the means of obtaining a large variety of information necessary for monitoring the health of astronauts on missions to the space station, the moon or beyond. Current advances in flow cytometry are primarily in the area of new probes for measuring new attributes of cells, chromosomes and molecules. Hence, in addition to the measurements briefly described here, new capabilities are being continuously added to the list of measurements routinely made by flow cytometers.

#### Acknowledgements

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TABLE 1: Cellular properties or constituents measured<sup>a</sup> with fluorescent dyes by flow cytometers.

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DNA  
RNA  
Protein  
Enzyme levels  
Cell surface antigens  
Mitochondria  
Membrane potential  
Cellular pH  
Mitochondria  
Chloroplasts

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Footnote:

a. References to details of these measurements can be found in Steinkamp, 1984; Braylan, 1983; and Melamed, 1979.

**Table 2: Nonfluorescence measurements<sup>a</sup> made with flow cytometers**

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Fluorescence polarization  
Time correlation  
Energy transfer  
Pulse widths  
Pulse risetimes  
Axial light loss

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**Footnote**

a. References to details of these measurements can be found in Steinkamp, 1984; Braylan, 1983; and Melamed, 1979.

## FIGURE LEGENDS

Figure 1. Schematic drawing depicting hydrodynamic focusing. The sample (white) is surrounded and focused by the sheath fluid (black) as it flows downward. The quartz walls of the flow cell are striped. The probe volume at the intersection of the focused laser beam and sample stream is on the order of  $10^{-12}$  liters.

Figure 2. Histogram of cellular DNA content. This histogram is the result of measuring the fluorescence emitted by a DNA specific dye as 75,000 cells pass through a flow cytometer. The peak at approximately channel 27 is due to cells in  $G_1$ . The peak at channel 55 is due to cells in  $G_2+M$ . The cells synthesizing DNA fall in between the two peaks.

Figure 3. Contour representation of a two dimensional histogram of forward and right angle light scatter from whole blood. The labeled islands are identified as being due to lymphocytes, monocytes, polys, and red blood cells. This figure demonstrates that a number of cell types can be identified on the basis of light scatter alone.

Figure 4. Contour representation of a two dimensional histogram of green

fluorescence (proportional to monoclonal antibody Leu 2a labeling) and red fluorescence (proportional to monoclonal antibody Leu 4 labeling). Of the cells which are Leu 4 positive indicating that they are T cells, 24% are Leu 2a positive which indicates that they are T suppressor cells.

Figure 5. Scanning electron micrograph of small (0.25 micrometer) fluorescent antigen coated microspheres bound to large (10 micrometer) antibody coated microspheres by antigen-antibody interactions.

Figure 6. Immunofluorescence displacement curve. The percent of the maximum fluorescence measured with no antigen present is plotted versus the concentration of antigen present. For this particular experiment, the detection limit for horse radish peroxidase was  $10^{-12}$  molar.

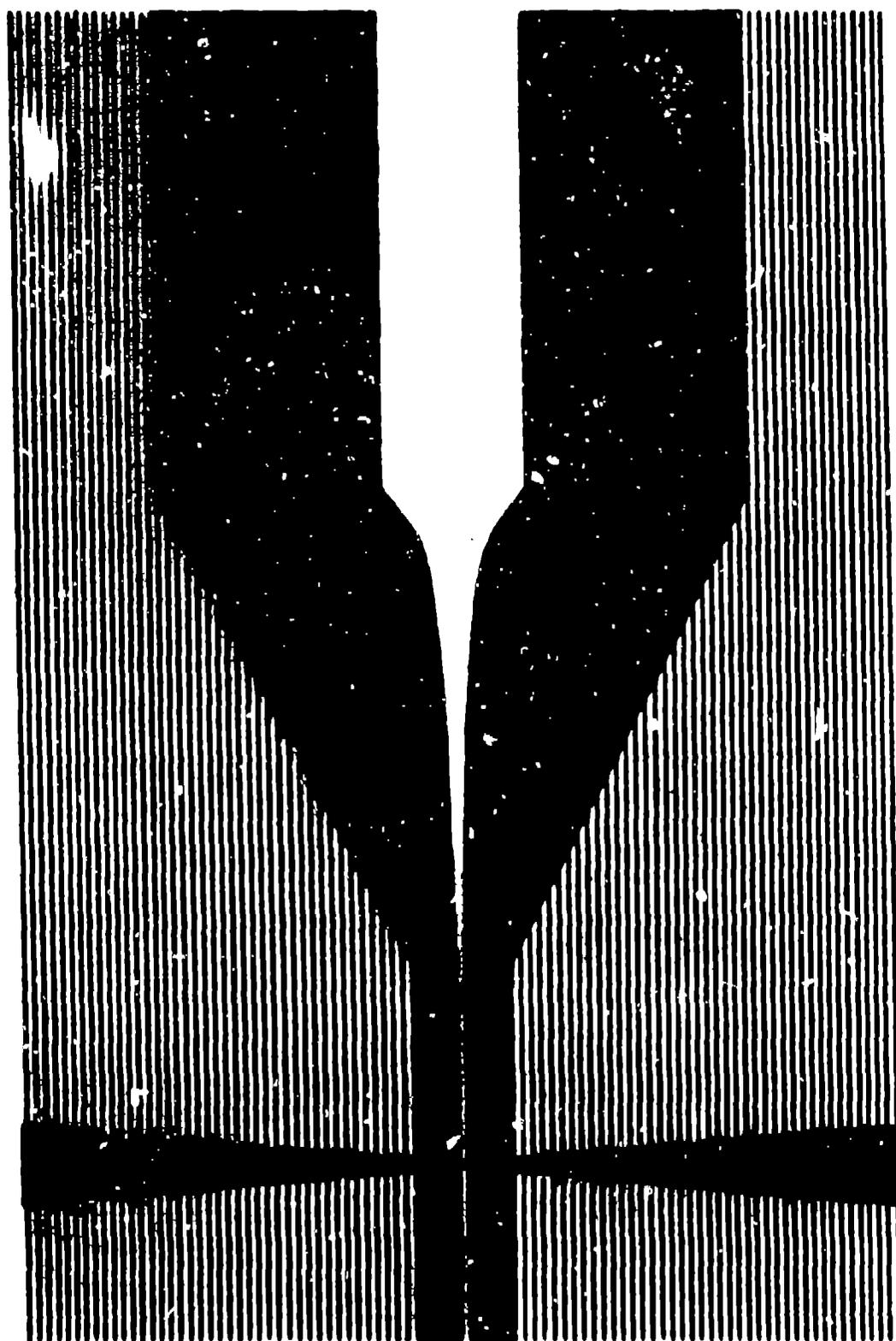


FIGURE 1

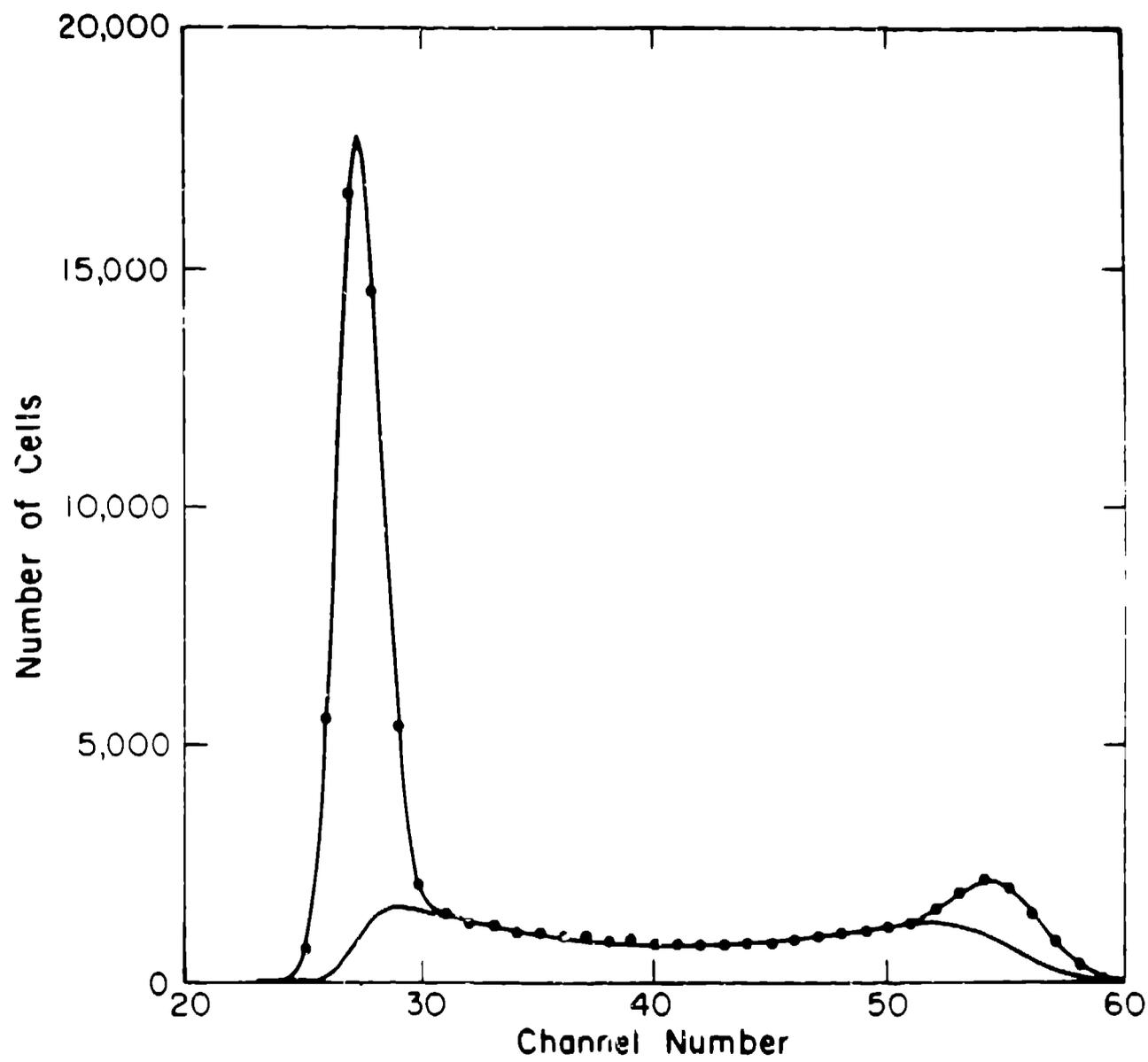


FIGURE 2

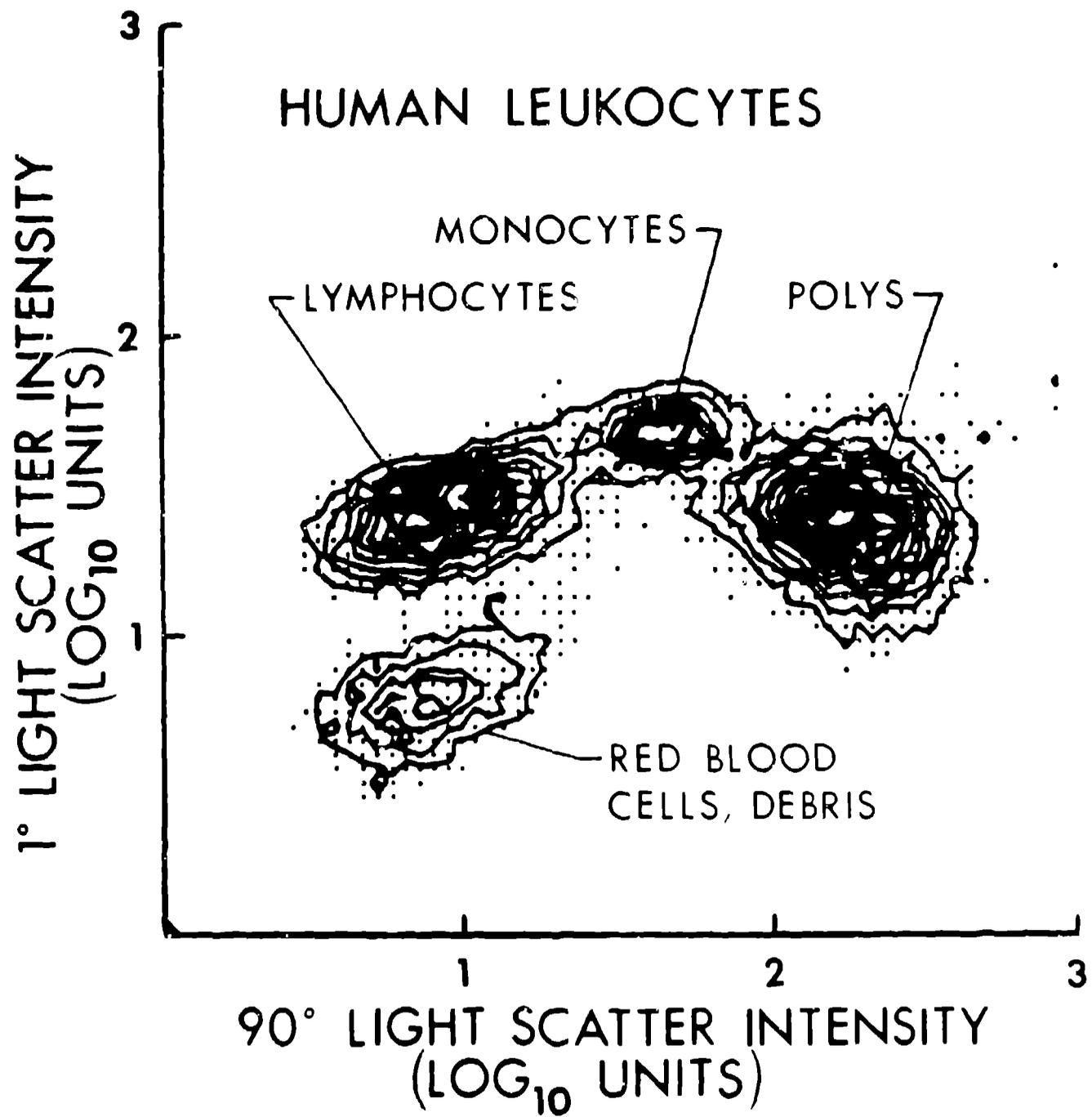


FIGURE 3.

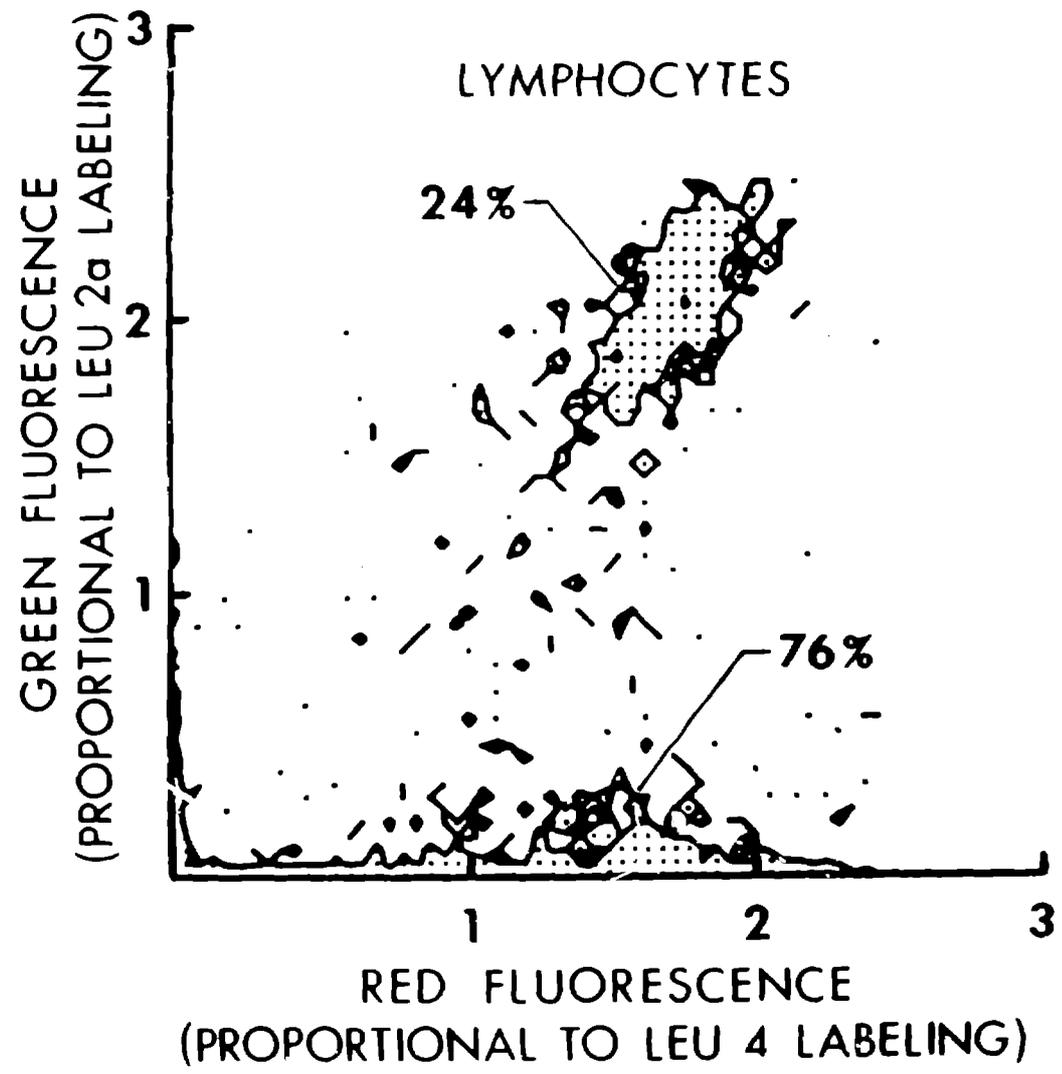


FIGURE 4

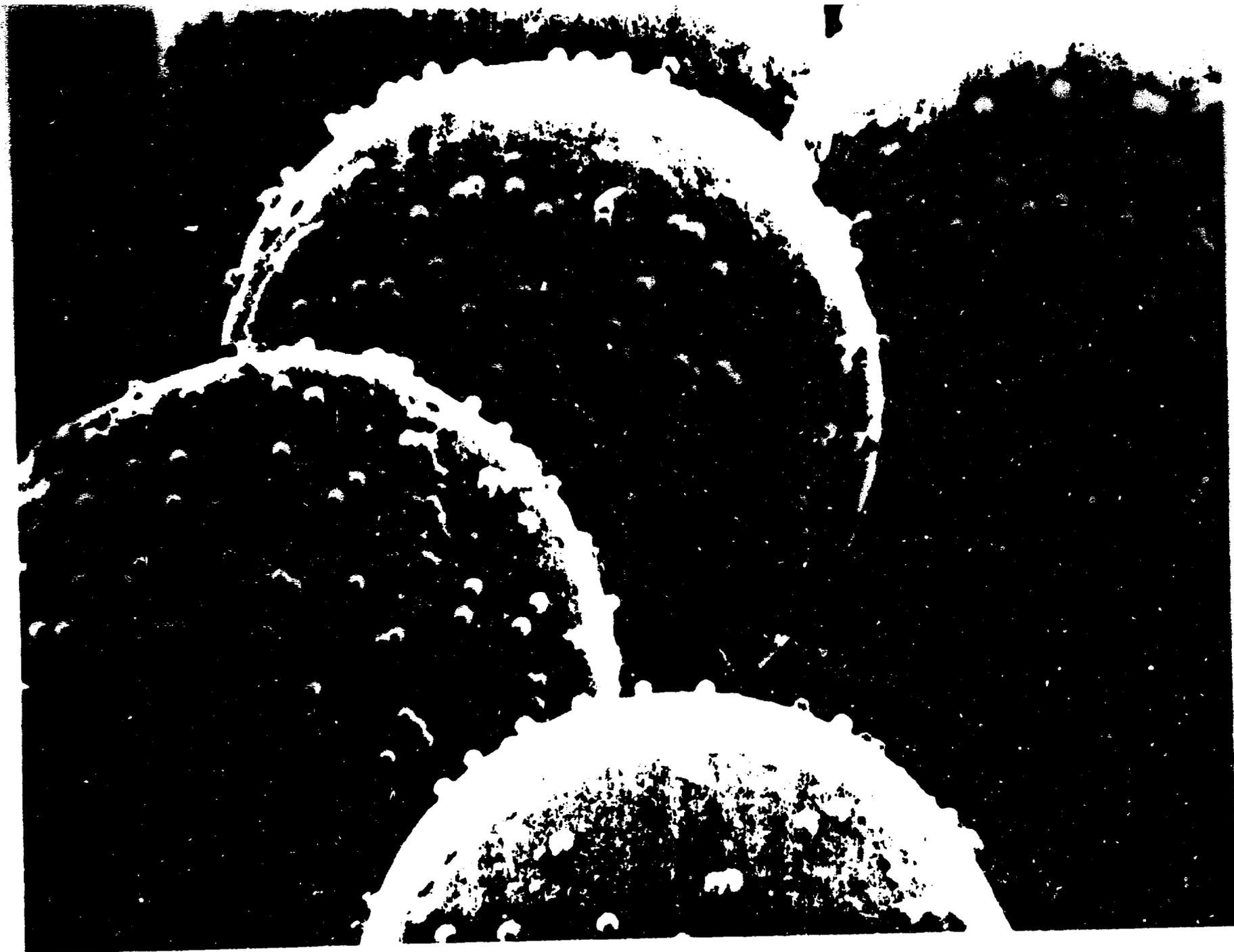


FIGURE 5

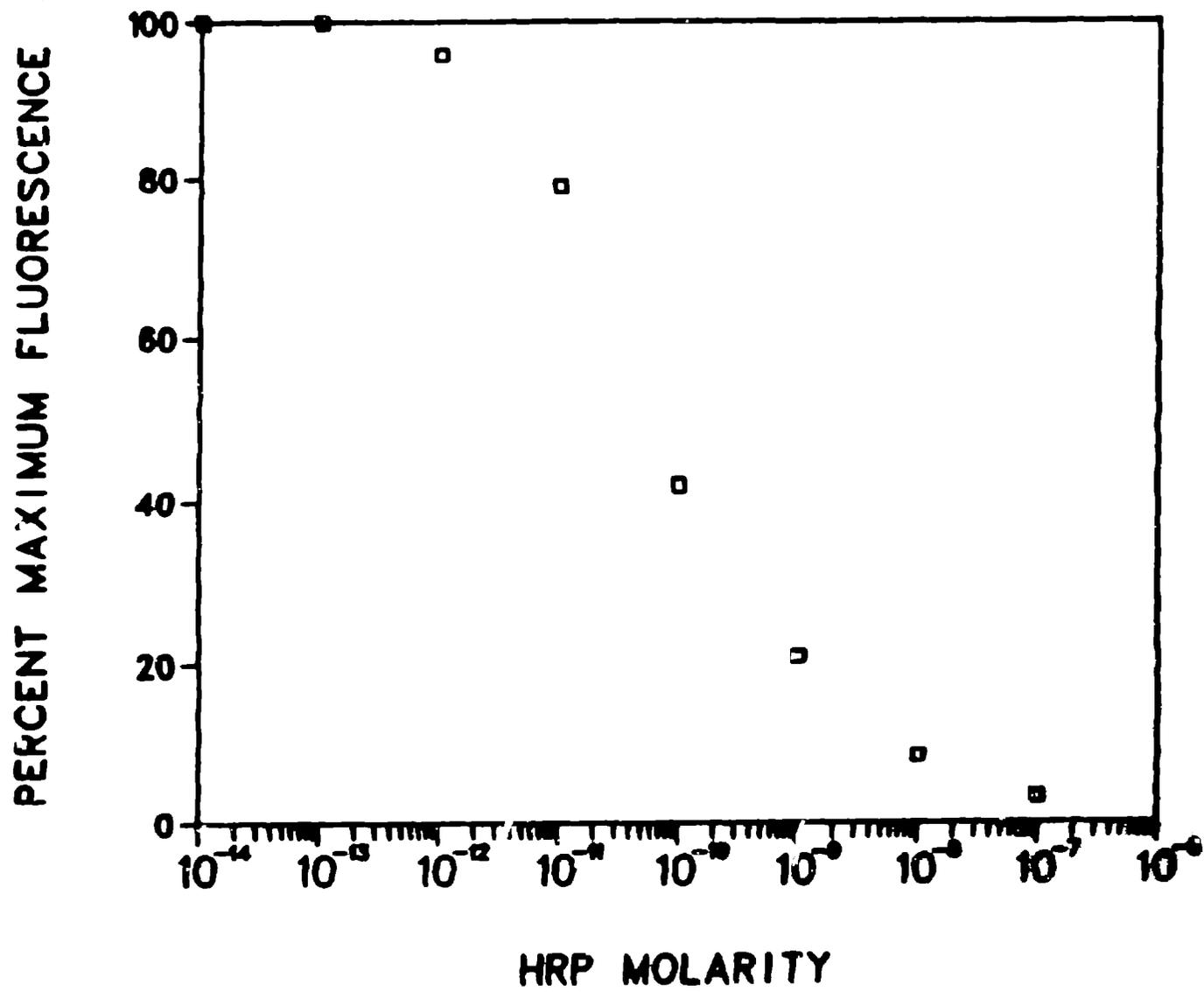


FIGURE 6