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LYMPHOCYTES IN A HYPOTONIC ENVIRONMENT: A FLOW CYTOMETRIC STUDY

by

LORI M. HOGG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **LYMPHOCYTES IN A HYPOTONIC ENVIRONMENT: A FLOW CYTOMETRIC STUDY** submitted by **Lori M. Hogg** in partial fulfilment of the requirements for the degree of **Master of Science** in **Experimental Pathology.**

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ABSTRACT

Exposure to a hypotonic environment constitutes a physiologic stress. How cells respond to that stress is an important question in cryobiology, because dilution of the liquid medium surrounding cells occurs during and after thawing. In this study, the effects of exposure to a hypotonic environment on human lymphocytes were characterized using flow cytometry.

A population of cells may be subdivided into surviving (live) and nonsurviving (dead) subpopulations following exposure to a stress. Live and dead cells are easily distinguished by flow cytometry, allowing each subpopulation to be characterized separately. This made possible four areas of focus: (1) what variables affect lymphocyte survival in a hypotonic environment? (2) what changes can be detected in lymphocytes prior to cell death? (3) what changes can be detected in surviving cells? and (4) how is lymphocyte volume affected by exposure to hypotonic stress?

Exposure to severe dilution was found to be more detrimental to lymphocyte survival at 25°C than at 0°C. Exposure to less dilute solutions, however, was more detrimental at the lower temperature. This observation implies that the mechanism of cell death may be different in the two tonicity ranges or at the two temperatures, and has implications for the design of

thawing protocols.

Changes in lymphocytes which were observed prior to cell death included loss of FALS intensity and loss of membrane potential. Loss of cell volume was observed as a late event, following cell death.

Lymphocytes which survived a dilution stress showed loss of intracellular ions, as indicated by loss of both volume and membrane potential.

Most lymphocytes regulate volume in a hypotonic environment at 25°C but not at 0°C. Flow cytometry allows the discrimination of volume-regulating from non-regulating cells by up to three intrinsic parameters.

Flow cytometry is demonstrated as a useful tool in the investigation of the responses of cells to alterations in their environment.

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To my family and Jeff, for helping me to hang in there.

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LIST OF ABBREVIATIONS

ADC	Analog to Digital Converter
BSA	Bovine Serum Albumin
CPD-1	Citrate-Phosphate-Dextrose-1
CVA	Coulter Volume Apparatus
$DiOC_5(3)$	Dipentyloxacarbocyanine
$DiSC_3(5)$	Dipropylsulfacarbocyanine
DMSO	Dimethylsulfoxide
EB	Ethidium bromide
FALS	Forward Angle Light Scatter
FC	Flow Cytometric
FCS	Fetal Calf Serum
FDA	Fluorescein diacetate
HEPES	4-(2 hydroxyethyl)-1 piperazineethane-sulfonic acid, sodium salt.
LPS	Lipopolysaccharide
90LS	Ninety-degree light scatter
PBS	Phosphate Buffered Saline
PHA	Phytohemagglutinin
PMT	Photomultiplier Tube
RPMI	Roswell Park Memorial Institute (place of origin of RPMI series medium)
SEM	Standard Error of the Mean

CHAPTER ONE

INTRODUCTION

The objective of this thesis is to characterize the effects of exposure to a hypotonic environment on human peripheral blood lymphocytes through the use of flow cytometric techniques.

When the environment of a cell is altered in a manner which constitues a physiologic stress, a cell can respond in one of three ways: it may die, it may survive altered in some way, or it may survive unchanged. A population of cells may be made up of several subpopulations which vary in their response to that stress.

For the purpose of this study, cell death may reasonably be defined as loss of membrane integrity. Survival is therefore defined as the maintenance of cell membrane integrity after exposure to a stress.

Using the above definition, dead and surviving cells are easily distinguished using flow cytometric techniques, allowing four fundamental questions to be addressed: what variables affect lymphocyte survival in a hypotonic environment; what changes can be detected in lymphocytes prior to cell death; what changes can be detected in surviving cells, and; how is lymphocyte volume affected by exposure to hypotonic stress?

To answer these questions, experimental protocols

were designed to expose lymphocytes to environments which varied according to degree of hypotonicity, duration of exposure, and temperature. Changes in optical properties, volume, and membrane potential were measured by flow cytometry.

CHAPTER TWO

LITERATURE REVIEW

2.1 RELEVANCE OF HYPOTONIC STRESS TO CRYOBIOLOGY

The response of cells to a hypotonic environment is a relevant issue in cryobiology because cells are exposed to dilution stresses during and after thawing.

When cells are frozen and thawed, a variety of stresses may exert their effects. These include the stress of temperature reduction (cold shock), the presence of ice inside the cell, and the stresses associated with the concentration of solutes in the extracellular fluid (solution effects). Exposure to a hypotonic environment constitutes a dilution stress.

Dilution stress has been identified as the most important post-thaw variable affecting cell survival after freezing (Farrant, 1980). Farrant points out that examination of the conditions for either progressive damage or repair of cells after storage is perhaps the most neglected area of cryobiology.

Dilution stress during cryopreservation occurs during thawing, as a consequence of the melting of extracellular ice, and after thawing, during removal of cryoprotectants (Farrant, 1980). Both the rate (Pegg, 1970) and the temperature (Thorpe, Knight and Farrant, 1976) at which the removal of cryoprotectant takes place have been identified as important variables affecting

post-thaw survival.

2.2 THE EFFECTS OF HYPOTONIC STRESS ON LYMPHOCYTES

Studies of the effects of hypotonic stress on lymphocytes over the past 20 years have reiterated a common theme: that lymphocytes vary in their susceptibility to hypotonic stress. Factors which may influence the observed heterogeneity of response to hypotonic stress are: cell type, state of differentiation, and disease state.

Thompson, Bull, and Robinson, (1966) showed a separated lymphocyte population to be composed of a spectrum of cell types which vary in their susceptibility to sudden death by shock treatment. A 30 sec. exposure to distilled water resulted in recovery of only 50% of the original cell population, and a further 10% showed gross morphological damage. The remaining lymphocytes showed resistance to subsequent hypotonic exposure, but did not respond to stimulation by phytohaemagglutinin (PHA).

Butterworth (1971) examined the hypotonic sensitivity of murine lymph node, thymus, and peripheral blood lymphocytes, and found that thymocytes were more sensitive than lymph node and peripheral blood lymphocytes. Butterworth suggested that the functional change occurring when cells are immunologically primed

could be accompanied by a membrane change which confers increased osmotic tolerance.

Thompson, Robinson, and Wetherley-Mein (1966) found that circulating lymphocytes in patients with chronic lymphocytic leukemia (CLL) consisted of two populations: adherent and non-adherent to polystyrene beads. The latter population was found to have properties similar to normal lymphocytes in terms of resistance to death by hypotonic shock. The adherent cells, in contrast, were abnormally resistant to hypotonic shock.

From a cryobiological perspective, extensive studies on the responses of murine lymphocytes to hypotonic stress have been carried out by Strong (Strong et. al, 1974; Strong, 1976). These studies compare PHA-responsive to lipopolysaccharide- (LPS) responsive lymphocytes. In the mouse, PHA responsive cells are T cells (Blomgren and Svedmyr, 1971) and lipopolysaccharide (LPS) responsive cells are B cells (Peavy, Adler, and Smith, 1970).

When compared to murine B cells, murine T cells are more susceptible to hypotonic stress and require a much shorter duration of exposure before damage is evident. Pretreatment of murine lymphocytes in hypotonic medium prior to freezing sensitizes murine T cells to subsequent freezing damage to a greater extent than B cells. Furthermore, murine T cells are more susceptible

to the rate of post-thaw dilution than are B cells.

2.3 VOLUME REGULATORY RESPONSE OF LYMPHOCYTES IN HYPOTONIC MEDIA

Despite reports that lymphocyte volume varies linearly with extracellular osmolality over a wide range (Law et al., 1983) there is considerable evidence to the contrary. A wide variety of nucleated mammalian and avian cells, including lymphocytes, have been shown to have the ability to regulate their volumes in hypotonic media (see Kregenow, 1981 for review).

Bui and Wiley (1981), found that human lymphocyte volume regulation in hypotonic media occurs largely by a passive loss of cell K^+, with accompanying exit of water. They postulated that the increased K^+ permeability is carrier-mediated.

Cheung et al. (1982) found that the K^+ permeability change was bidirectional, in that both efflux and influx of K^+ were enhanced. The volume change, therefore, would be expected to depend on the extracellular K^+ concentration. This was indeed found to be the case, as they were able to confirm the observation of Ben-Sasson et al. (1975) that in high K^+ medium, shrinkage is absent and secondary swelling occurs.

Grinstein et al. (1982) examined the role of Ca^{2+}

in the volume regulatory response. Lew and Ferriera (1978) had shown that Ca^{2+} plays a role in K^+ efflux during volume regulation in duck erythrocytes. Bui and Wiley (1981) had been unable to substantiate such a role in the case of human lymphocytes.

Grinstein et al., however, found that effects similar to those induced by hypotonic stress could be produced by raising the intracellular Ca^{2+} level with the Ca^{2+} ionophore A23187. Quinine, a known inhibitor of Ca^{2+}-activated pathways in other systems, blocked the volume regulatory response. In accordance with Bui and Wiley, however, Grinstein et al. found that extracellular Ca^{2+} concentration had no effect on K^+ permeability.

These results led Grinstein et. al. to propose the hypothesis that cell swelling triggers the release of Ca^{2+} from intracellular stores, such as mitochondria and endoplasmic reticulum, which results in activation of a Ca^{2+}-dependent K^+ channel.

Grinstein et al. (1982a) tested the assumption, based on data from red blood cells, that anion conductivity in human lymphocytes is initially high and is unaffected in the hypotonic response. This proved not to be the case, as they found direct evidence that Cl^- conductivity is initially low, and is increased by hypotonic exposure.

Sarkadi et al. (1984), using the channel-forming

ionophore gramicidin to shunt cation transport so that anion transport alone would limit the volume response, were able to show that the Cl^- and K^+ conductance pathways are independent, and that the K^+ pathway has properties similar to the Ca^{2+}-activated transport pathways observed in various cell membranes.

Sarkadi et al. (1984a) examined the kinetics of the opening and closing of the anion and cation pathways during the volume regulatory response. They found that the opening of the Cl^- pathway was triggered by the increase in volume: a threshold volume of 1.15x isotonic was required for the Cl^- channel to open. If the volume of a swollen cell was decreased below the threshold volume, the Cl^- transport pathway was inactivated. Activation and inactivation of the Cl^- pathway are independent of the relative volume changes and of the actual cellular ionic concentrations. In contrast, the increase in K^+ conductance was found to be graded in response to the increase in cell volume.

The opening of specific ion channels in response to deviations from normal osmotic conditions is especially interesting in light of the participation of ion channels in cellular activation. K^+ channels are known to play an important role in the stimulation of lymphocytes by mitogens (Quastel and Kaplan, 1970; Kay, 1972). There is also strong evidence (Truneh et al.,

1985) for the involvement of Ca^{2+} in lymphocyte activation.

CHAPTER THREE

INTRODUCTION TO FLOW CYTOMETRY

3.1 THE FLOW CYTOMETER

A flow cytometer is an instrument which measures optical properties of single cells in suspension as they interact with a laser light source. The cells are hydrodynamically focussed (Spielman and Goren, 1968), so that they pass through the laser one by one. Light scattered and fluoresced by the cells is detected by photomultiplier tubes (PMT's). Light intensity is transduced to voltage pulses by the PMT's. The voltage pulse is then integrated and converted to a digital signal by a 256-channel analog-to-digital converter (ADC). The data is accumulated in computer memory as 256-channel histograms of cell number versus light intensity.

Data can be displayed either as a one parameter histogram, as shown in Fig. 3.1.1, or as a two parameter histogram, as shown in Fig. 3.1.2. A one parameter histogram actually displays two varibles; the second, in the vertical plane, being frequency or number of cells. Similarly, a two parameter histogram displays three variables. The terms, "one parameter," and "two parameter," are conventionally accepted in the the field of flow cytometry (Shapiro, 1985).

Any one parameter, such as low angle light scatter

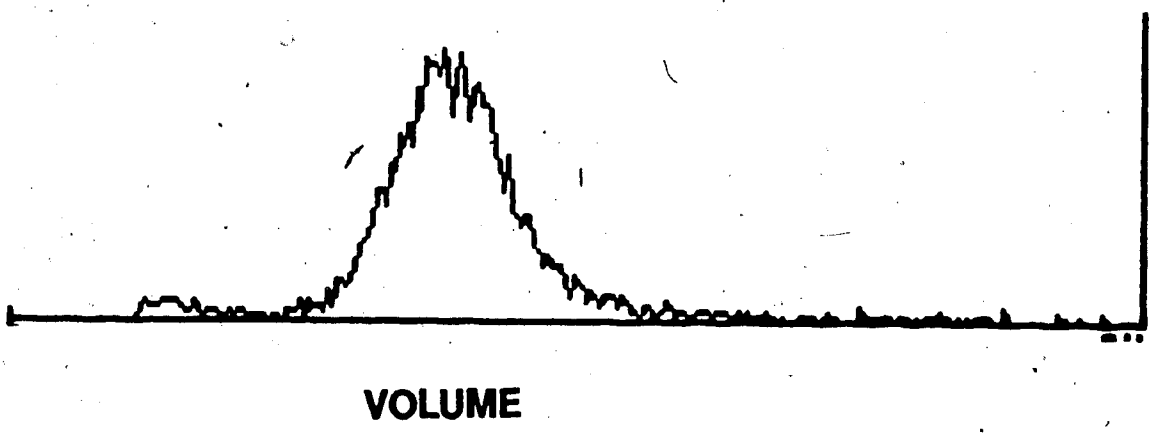

FIGURE 3.1.1 One parameter histogram of volume (horizontal axis), versus number of cells (vertical axis).

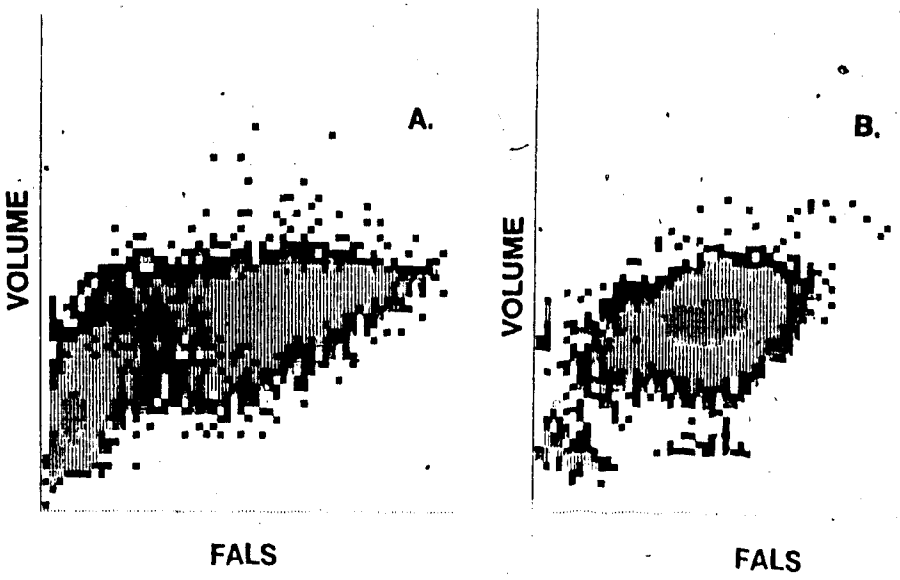

FIGURE 3.1.2 Two-parameter histograms of FALS (horizontal axis) versus volume (vertical axis). The third dimension, represented by contour lines, is number of cells. Before treatment (A), and after treatment (B).

or fluorescence at a particular wavelength, can be used to define a gating window within which to measure any other parameter. Gating windows are one way to investigate the relationship between two parameters.

Two parameter histograms (Fig. 3.1.2) can also be used to graphically show the relationship of two parameters. The horizontal and vertical axes in the plane of the paper each represent one of two parameters. A third dimension, out of the plane of the paper, is implied by the contour lines, which represent the number of cells at each level. Two parameter histograms may also be measured within gating windows defined by other parameters.

Two parameter histograms are very helpful in monitoring changes that are occurring simultaneously in more than one variable. For instance, inspection of Fig. 3.1.2 (A) reveals that the before-treatment population has a uniform distribution in terms of FALS and volume. The roughly circular distribution of the population in two planes of the paper is the consequence of plotting two normally distributed variables orthoganally with respect to each other. In contrast, the distribution of the after-treatment population (Fig. 3.1.2 B) is no longer uniform: two distinct subpopulations are now evident. One subpopulation has diverged down and to the left (both FALS and volume have

decreased), whereas the other subpopulation has diverged to the right while maintaining the same vertical level (FALS has increased while volume remains the same).

The capacity to simultaneously measure several variables for each individual cell in a sample of thousands of cells makes the flow cytometer a very powerful tool. The capacity to illustrate how each variable is related to the other through the use of two parameter displays and gating makes this instrument truly unique.

3.2 APPLICATION AND THEORETICAL ASPECTS

Flow cytometry has found its widest application in the qualitative definition of positive and negative subpopulations with regard to a particular fluorescent antibody (Loken and Stall, 1982). However, the scope of applications is rapidly expanding (see Shapiro, 1983 for review).

The following discussion presents a brief introduction to the theoretical basis of the assays used in this work. For convenience, I have used Shapiro's (1985) classification of cellular flow cytometric parameters as either intrinsic or extrinsic, based upon whether or not they can be measured without the use of reagents. Intrinsic parameters include light scatter and volume. Extrinsic parameters are those which depend upon the application of an extrinsic dye or

label, and the light emitted is fluorescent; ie., emits at a wavelength other than the excitation wavelength of the laser.

3.2 a) Intrinsic Parameters

Light Scatter

Although forward, or low angle light scatter is often used as an indicator of cell-size, it is actually a complex function of several variables. A near-linear relationship between size and forward scatter is true only within a certain range of diameters and only for smooth spherical objects where size is the only variable (Mullaney and Dean, 1970). The theoretical basis for the dependency of scatter on the refractive and reflective properties of the particle or cell are reviewed by Salzman et al., 1979, and Salzman, 1982.

Forward angle light scatter (FALS) is routinely used to exclude dead cells from analysis (Loken et al., 1976). Dead cells have characteristically low intensity FALS, and gated analyses of scatter distributions have shown that in mixtures of live and dead cells, the low intensity peak corresponds to cells which do not fluoresce when stained with the fluorogenic compound fluorescein diacetate (FDA) (Julius and Herzenberg, 1974; Loken and Herzenberg, 1975). However, reduced light scatter in the case of dead cells is not due to a reduction in cell size. Herzenberg and Herzenberg

(1978) suggest that changes in refractility between live and dead cells may be responsible for both the difference in appearances with phase contrast microscopy and light scatter intensity. The exact physical basis for this extremely convenient phenomenon remains obscure.

Ninety degree light scatter (90LS) is thought to be more closely related to internal structural characteristics of the cell than is scatter at low angles. Salzman (1982) estimates that 90LS response is roughly five times more sensitive to changes in refractive index than is FALS. Shapiro (1983) found 90LS was correlated with measurements of total protein content as estimated from the fluorescence of a protein-binding acid dye.

The combination of FALS and 90LS in two-parameter histograms has been found to be very useful in discriminating between different cell types. Salzman et al. (1975) used two-parameter light scatter to distinguish unstained lymphocytes, monocytes, and granulocytes in buffy coat preparations of human peripheral blood. MacDonald and Zaech (1982) used two parameter light scatter to discriminate between lymphocytes and lymphoblasts in mixed lymphocyte culture.

Visser et al., (1980) used two parameter light

scatter to show the sensitivity of both parameters to the osmolality of the suspending medium, using murine erythrocytes. At high osmolality, 90LS increased, and at low osmolality, decreased. This is interpreted with respect to the changes in refractive index which would result from water entering or leaving the cell. Forward scatter decreased when the cells were crenated in high osmolality solution, and increased when the cells became swollen spherocytes in low osmolality medium.

Volume

Coulter Electronics (Hialeah, Fla.) has recently (1985) made available the Coulter Volume Adapter (CVA) as an accessory to the Coulter EPICS flow cytometer, allowing simultaneous measurement of volume and photometric parameters. The CVA is based on a modification of the Coulter transducer (Coulter, 1953, 1955) which measures the changes in electrical resistance produced by a particle suspended in an electrolytic medium traversing a narrow aperature.

The theoretical aspects of electrical sizing are reviewed by Kachel (1979) and Grover et al. (1982). The change in resistance which occurs when a cell traverses the aperature is influenced by cell-specific and aperature-specific variables. The former are cell volume, shape, orientation, and the electrical resistivity of the internal milieu of the cell compared to the surrounding medium. The latter are the current

through the aperature, and the geometric dimensions of the aperature. The magnitude of the voltage pulse produced by the cell is the characteristic which most nearly exhibits proportionality to cell volume.

When the permeability of cell membranes is altered as a result of stress or damage, the electrical size of the cell may be expected to decrease (Grover et al., 1982a), since the internal milieu of the cell becomes more similar to the external medium. This phenomenon has been applied to measure the osmotic fragility of human erythrocytes (Gear, 1977) and to monitor early changes in membrane permeability associated with immune cell lysis (Reif et al., 1977).

3.2 b) Extrinsic Parameters

Membrane Potential

The use of lipophilic, cationic dyes as indicators of membrane potential has been reviewed by Shapiro (1985). Dipentyloxacarbocyanine, $DiOC_5(3)$, is a member of a class of compounds which distribute across the plasma membrane at concentrations dependent on the cytoplasmic membrane potential.

In their comparative study of 29 different cyanine dyes, Sims et al. (1974) emphasized the use of two, $DiOC_5(3)$ and dipropylsulfacarboycyanine, $DiSC_3(5)$, because of the large fluorescence changes exhibited by

these dyes in response to maximal hyperpolarization induced by the K^+ ionophore valinomycin, and because of their stability and lack of toxicity. $DiOC_5(3)$ has found particular application in the study of the membrane response of individual human neutrophils to stimulatory agents (Seligmann, Chused, and Gallin, 1981).

CHAPTER FOUR

METHODS AND MATERIALS

4.1 ISOLATION OF LYMPHOCYTES

Blood was collected from healthy donors by the Canadian Red Cross Blood Transfusion Service in donor bags with citrate-phosphate-dextrose-glycine (CPD-1) as the anticoagulant. The buffy coat layer was separated from the whole blood by centrifugation at 700xg for 10 min. in a Sorvall RC-3 centrifuge. The buffy coat was diluted 1:1 (v/v) with isotonic PBS and 30 to 35 ml of the diluted mixture were layered onto 15 ml of Isolymph (sodium diatrizoate and Ficoll 400; density 1.077 g/ml; Gallard-Schlesinger, Carle Place, N.Y.) in four 50 ml centrifuge tubes. The tubes were centrifuged at 400xg for 40 min. and the mononuclear layer was collected from the interface (Boyum, 1968).

The cells were then washed twice at 400xg for 5 min. with cold PBS/BSA and resuspended in RPMI 1640 (Gibco) with 20% FCS (Flow Laboratories, Inc.) at a concentration of 20 x 10^6 cells/ ml. Twenty ml of the cell suspension were placed in each of four plastic tissue culture dishes (100x20 mm Falcon 3003) and incubated at 37°C in 5% CO_2 for 90 min. The non-adherant lymphocytes were removed by gently swirling and then pouring off the suspension, and rinsing the dishes gently with warm PBS/BSA. Adherant monocytes were left

behind. The lymphocytes were then washed twice for 5 min. at 300xg to remove platelets. Contaminating red cells were not lysed.

Lymphocytes not used within three hours of separation were suspended in RPMI 1640 plus L-glutamine (20 mM) (Gibco) with 25 mM HEPES buffer, 10% FCS and 1% penicillin/streptomycin (Gibco), at a cell concentration of 1 $x10^6$ cells/ml and incubated at 37°C, in a 5% CO_2 atmosphere. All lymphocytes were used within 24 hr of collection.

Immediately before use, lymphocytes were centrifuged and resuspended in isotonic PBS with 0.5% BSA (Miles Pentex) at a concentration of 1x 10^7 cells /ml.

4.2 STRESS PROTOCOLS

These procedures were performed at room temperature (25 +/- 2 °C) and on melting ice (0°C).

A shorthand is used throughout this thesis to denote tonicity of suspending media: osmotic strength is expressed as a decimal fraction of the isotonic osmolality. For example a 0.1x solution has one tenth the osmolality of an isotonic solution (0.1 x 300 mOsm/kg = 30 mOsm/kg).

4.2 a) Severe hypotonic stress for increasing time, followed by return to isotonic conditions.

Seven aliquots were prepared by adding 0.1 ml of cell suspension to 0.9 ml of distilled water (0.1x final concentration). After time intervals of 0.5, 1, 2, 3, 5, 7, and 10 min. the samples were returned to isotonicity by addition of 0.1 ml of 10x PBS. A control sample was diluted with isotonic PBS to the same final volume (1.1 ml).

4.2 b) Increasing hypotonic stress for a fixed time interval, followed by return to isotonic conditions.

Ten aliquots were prepared by adding 0.1 ml of cells to 0.9 ml of medium prepared to achieve dilutions of 0.1x to 1x isotonicity. After 5 min., 0.1 ml of the appropriate hypertonic medium were added to return the cell suspension to isotonicity. Details of the preparation solutions for this protocol are found in Appendix A.

4.2 c) Mild hypotonic stress for increasing time: no return to isotonic conditions.

This protocol was used to investigate volume regulation in lymphocytes.

One hundred µl of cell suspension were added to a mixture of 0.4 ml distilled water and 0.5 ml isotonic PBS (0.6x final concentration).

Flow cytometric (FC) analysis was performed at intervals for up to 20 min after dilution without

adjusting the tonicity of the medium.

4.3 FLOW CYTOMETRIC (FC) ANALYSIS

4.3 a) Equipment

FC analysis was performed using an EPICS V flow cytometer (Coulter Electronics, Hialeah, FLA.), equipped with a Coulter Volume Apparatus (CVA) and a dual laser. Only the argon laser (Innova 90 Series, Coherent Laser Products Division, Palo Alto, CA.) was used in this study. The excitation wavelength was set at 488 nm at all times. Laser power was controlled automatically at a constant output of 500 mW.

Details of optical filter setups, PMT high voltage and gain settings and gating parameters are included in Appendix B.

4.3 b) Data Analysis

Frequency histograms may be described by a number of statistics. The mean channel is the channel having the average intensity of that parameter in the population of cells measured. The peak, or modal, channel is the channel having the highest frequency. Differences in the mean and peak channels are indicative of deviations from a normal distribution of that parameter within the population of cells. In this study, both means and peaks were measured, but peaks were used to describe trends in the data. Peak values

were normalized as a percent of the isotonic, unstressed control peak.

4.4 MEASUREMENT OF INTRINSIC PARAMETERS: VOLUME, FALS AND 90LS

Experiments to determine the effects of hypotonic stress on lymphocyte intrinsic FC parameters were conducted in replicates of three, each using lymphocytes from a different donor, for each stress protocol at both $0^{o}C$ and $25^{o}C$. Flow cytometric analysis was carried out at room temperature for all samples, but the samples which had been stressed at $0^{o}C$ were held on ice until analysis.

The gain and high voltage settings were chosen to place the peaks of the isotonic control histograms approximately in the middle of the 256 channel range, so that both increases and decreases in the parameters could be monitored. To eliminate contaminating platelets, erythrocytes, and debris from FC analysis, data accumulation gates were set to exclude the lower channels in both FALS and volume histograms.

Peak values were obtained for 90LS as log values, and were converted to linear peak values using the formula:

$$\text{linear peak channel} = 2.56\ (10^{\log \text{peak channel}/85.3})$$

4.5 MEASUREMENT OF MEMBRANE POTENTIAL

The dye, 1,3-dipentyloxacarbocyanine, $DiOC_5(3)$, was used as an indicator of membrane potential (Waggoner, 1976). The dependence of dye fluorescence on cellular membrane potential is demonstrated in Appendix C.

$DiOC_5(3)$ (Molecular Probes) was prepared as a 1 mM stock solution in dimethyl sulfoxide, DMSO (Fisher), and stored protected from light at room temperature. A working solution was prepared by making a 1 in 75 dilution of stock solution with DMSO. Eight µl of the working solution were added to 1 ml of cell suspension, to give a final dye concentration of 106 nM. The total concentration of DMSO was not more than 0.15% (v/v).

When lymphocytes were stressed and then returned to isotonic conditions (protocols 4.2 a,b) the dye was added after return to isotonic conditions.

When lymphocytes were stressed at 0°C, the samples were allowed to warm to room temperature before adding the dye. The cells were incubated in the dark for 30 min before FC analysis, and the fluorescence remained stable for at least two hr. Control samples were analyzed at the beginning and end of a run of stressed samples to monitor any drift in fluorescence over time.

4.6 CELL VIABILITY INDEX

Cell death can reasonably be defined as irreversible loss of membrane integrity. Loss of membrane integrity, as indicated by vital stains such as ethidium bromide and fluorescein diacetate, is correlated with loss of FALS intensity (see Appendix D). By gating on FALS, live and dead cells can therefore be distinguished easily by flow cytometry and analyzed separately.

An accurate index of cell survival, however, should combine measurment of the number of cells which have maintained membrane integrity with the number of remaining intact cells, ie, those which have not lysed. This can be done by measuring the total number of cells in a given volume of the stressed sample, and comparing this to the number cells in the control. This proportion is then multiplied by the proportion of the intact cells which have maintained membrane integrity, to obtain lymphocyte survival (%).

To count cells in a given volume, an internal standard was used. An internal standard is a suspension of particles which differs in light scatter properties from lymphocytes, and which can therefore be added to a sample of lymphocytes and counted separately. For this study, the particles had to be unaffected by hypotonic media, and therefore, polystyrene beads (Coulter 15 µm

diameter microspheres, #6602707, Coulter EPICS Division, Hialeah, Fla.) were chosen. Gates were set on the 90LS distribution of the beads, which was outside of the range of lymphocytes, and a fixed number of bead counts was chosen as an endpoint for data accumulation.

CHAPTER FIVE

RESULTS

5.1 EFFECT OF HYPOTONIC STRESS ON LYMPHOCYTE SURVIVAL.

When lymphocytes were exposed to severe hypotonic stress (0.1x medium) for increasing duration at 25°C, survival was less than when exposure took place at 0°C (Fig. 5.1.1). Only 60% of cells survived a one min. exposure at 25°C. In contrast, 60% of lymphocytes survived a 3 min. exposure to 0.1x medium at 0°C.

Exposure to increasing dilution for a constant time, 5 min., (Fig. 5.1.2) showed that the detrimental effect of hypotonic stress on lymphocyte survival was greater at 25°C than at 0°C only in very dilute solutions (0.2x, 0.1x). In less dilute solutions (0.4x, 0.3x), lymphocytes showed enhanced survival when diluted at 25°C compared to those diluted at 0°C. Exposure to solutions less dilute than 0.5x had little effect on lymphocyte survival at either temperature.

5.2 CHANGES ASSOCIATED WITH LOSS OF MEMBRANE INTEGRITY.

Two parameter histograms allow determination of the sequence of events associated with loss of membrane integrity, as indicated by loss of FALS.

In Fig. 5.2.1 (A) the unstressed control shows a

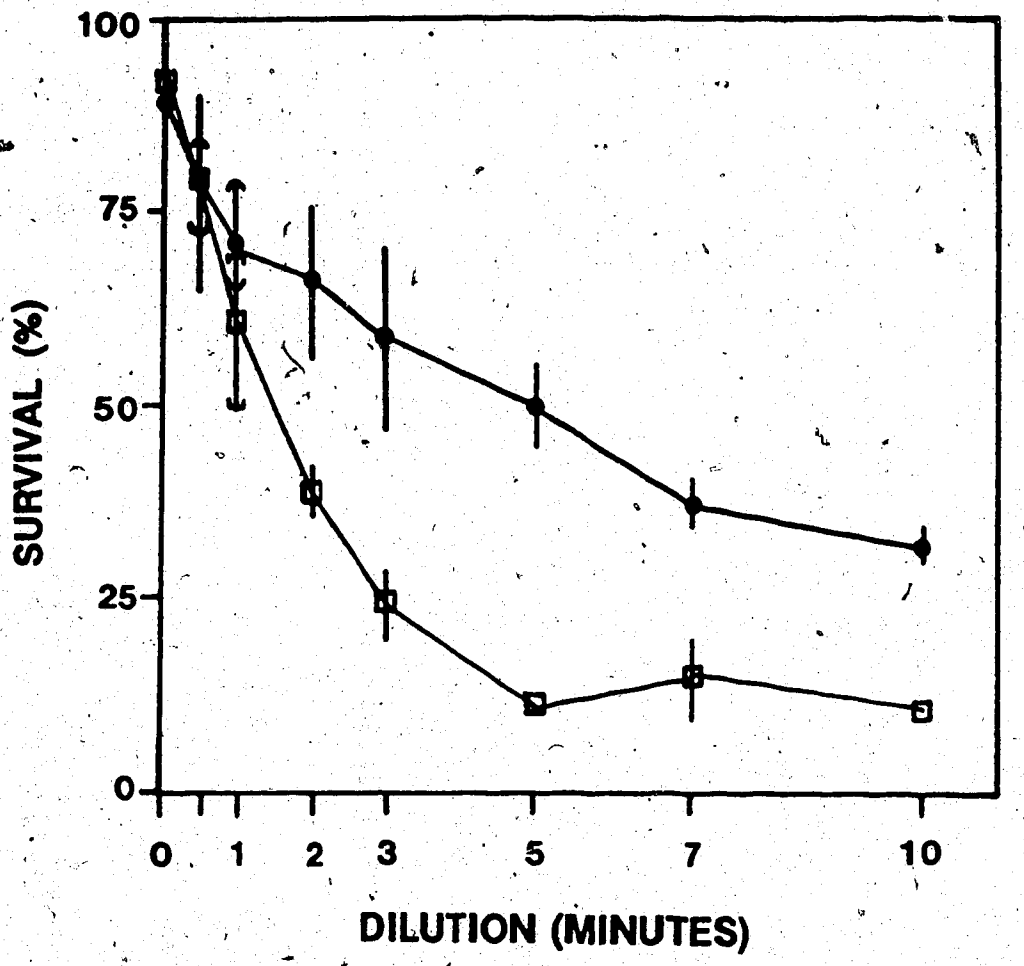

FIGURE 5.1.1 Effect of severe dilution for increasing time on lymphocyte survival (see Chapter 4.6 for explanation of cell viability index), at 25°C (□) and 0°C (●).

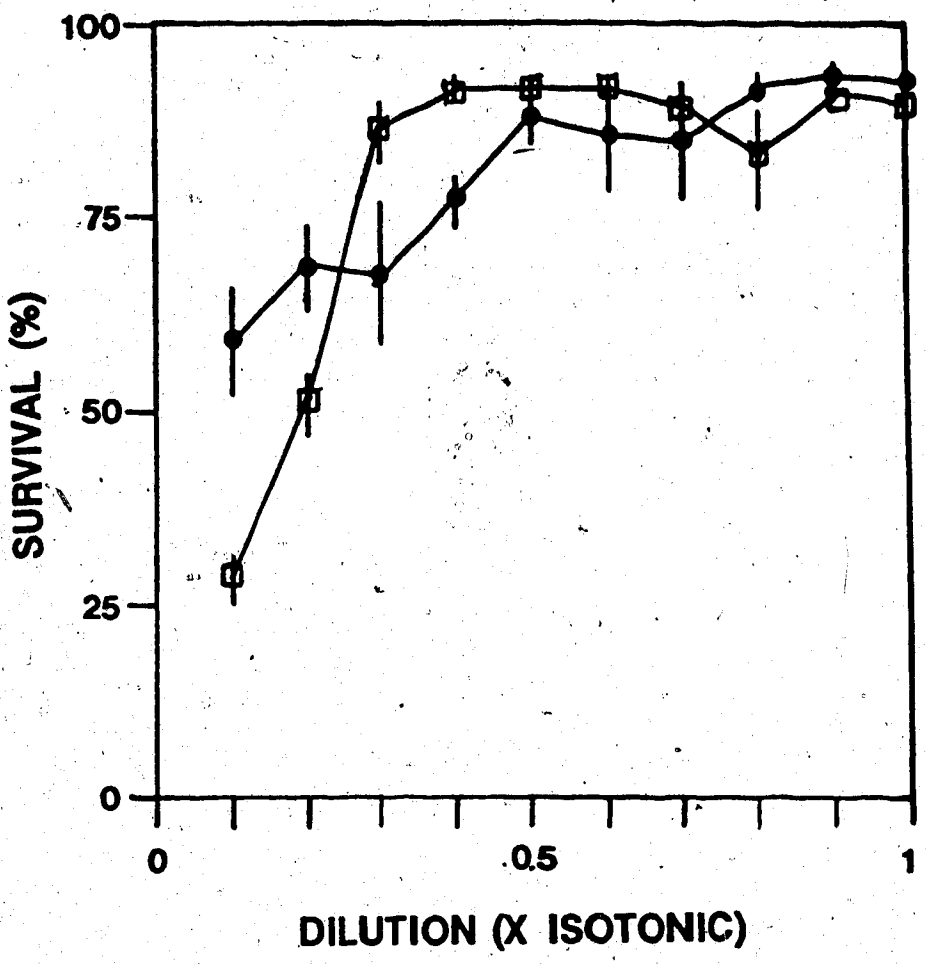

FIGURE 5.1.2 Effect of increasing dilution for 5 min. on lymphocyte survival (see Chapter 4.6 for explanation of cell viability index), at 25°C (□) and 0°C (●).

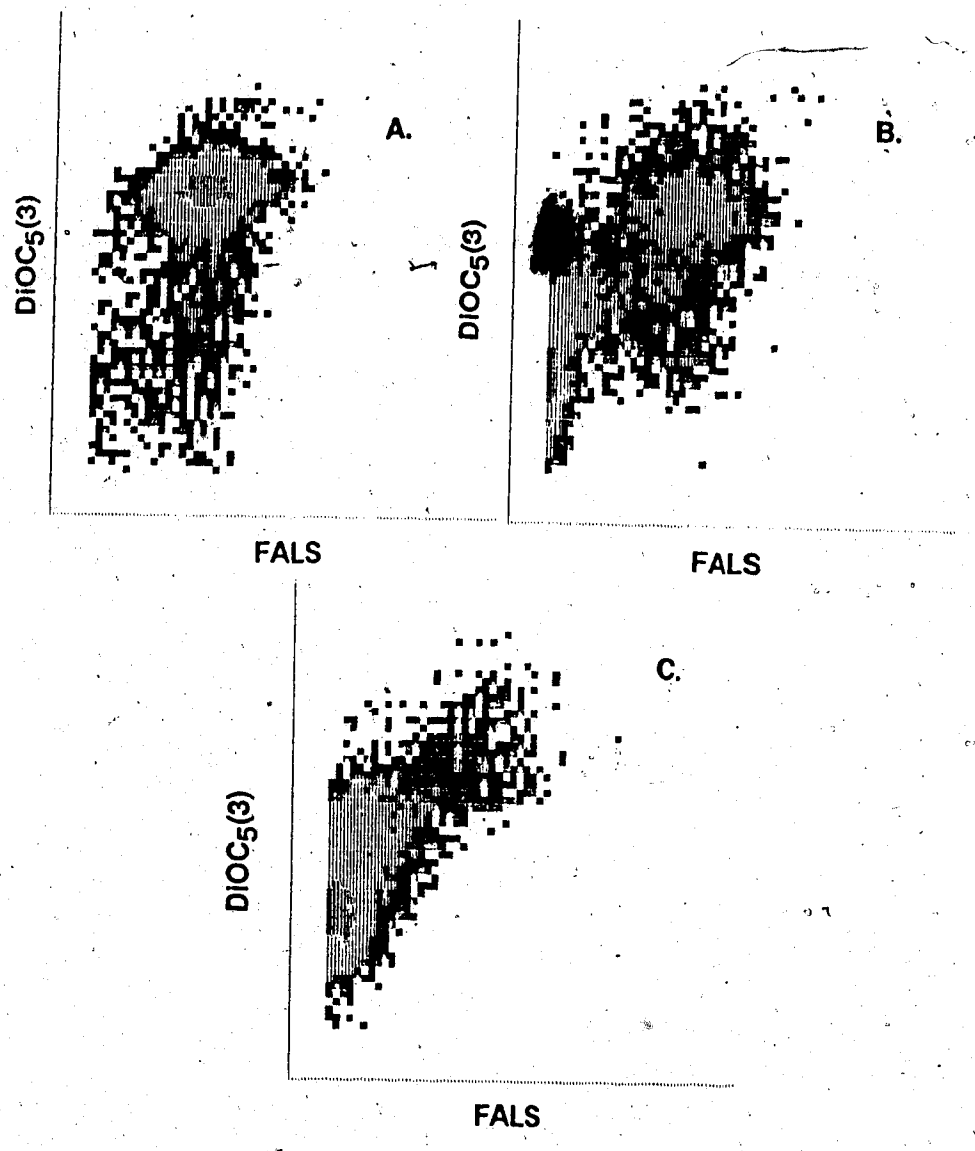

FIGURE 5.2.1 Two parameter histogram of FALS (horizontal axis) versus $DiOC_5(3)$ fluorescence (vertical axis) for lymphocytes exposed to 0.1x medium at 25°C for A) 0 min., B) 2 min., C) 10 min.

population which has a FALS (horizontal axis) distribution which is narrow (confined to the left half of the horizontal scale) and appears to be normally distributed (the number of cells on each side of the peak is approximately equal). The distribution of membrane potential (vertical axis) in this population is wider and skewed towards the top end of the scale (more cells have greater membrane potential).

After a 2 minute exposure to stress (Fig. 5.2.1 B) two subpopulations are evident: one which is the same as the control population, and one which has shifted to the left on the horizontal axis (loss of FALS intensity) and down on the vertical axis (loss of membrane potential). The diagonal shift indicates that both parameters are changing simultaneously. Loss of FALS was therefore coincident with loss of membrane potential.

By 10 min. of exposure (Fig. 5.2.1 C) almost all cells show decreased FALS and membrane potential. A few cells, however, retain the characteristics of the original, unstressed population.

Fig. 5.2.2 shows how volume changed with respect to membrane potential. Exposure at $0^{o}C$ is used to illustrate these events, because the time scale is expanded. After a 2 min. exposure to severe stress at $0^{o}C$ (Fig. 5.2.2 C) some cells had lost membrane

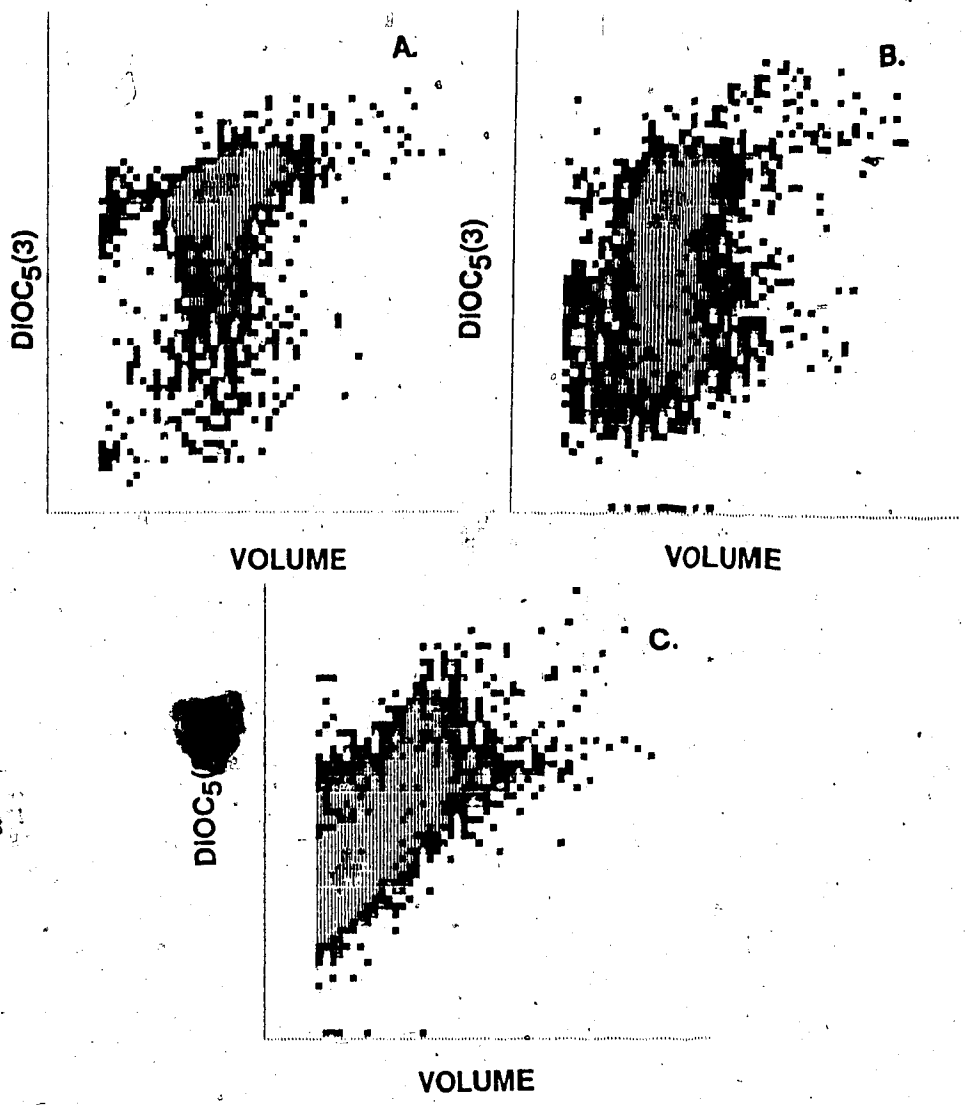

FIGURE 5.2.2 Two-parameter histograms of volume (horizontal axis) versus $DiOC_5(3)$ fluorescence (vertical axis) for lymphocytes exposed to 0.1x medium for A) 0 min., B) 2 min., C) 10 min., at 0°C.

potential (shift down on vertical axis), but most of these depolarized cells had maintained isotonic volume (horizontal axis). By 10 min of exposure, most cells had lost both membrane potential and volume. Cells which maintained volume were not depolarized.

In summary, loss of FALS intensity was observed to coincide with loss of membrane potential. Loss of volume, in contrast, occurred following loss of membrane potential, and is therefore a late event following cell death.

5.3 CHANGES DETECTED IN SURVIVING CELLS

5.3 a) Effect of severe hypotonic stress for increasing time, return to isotonic.

When exposed to very dilute medium (0.1x), surviving lymphocytes showed decreased volume with increasing duration of stress at 25°C (Fig. 5.3.1 A). In contrast, when stressed at 0°C, surviving cells showed almost no change in volume (Fig. 5.3.1 B).

Surviving lymphocytes showed increased FALS intensity with increasing duration of stress at 25°C (Fig. 5.3.2 a). In contrast, when stressed at 0°C, little change in FALS intensity was observed (Fig. 5.3.2 b).

An increase in the intensity of 90LS with

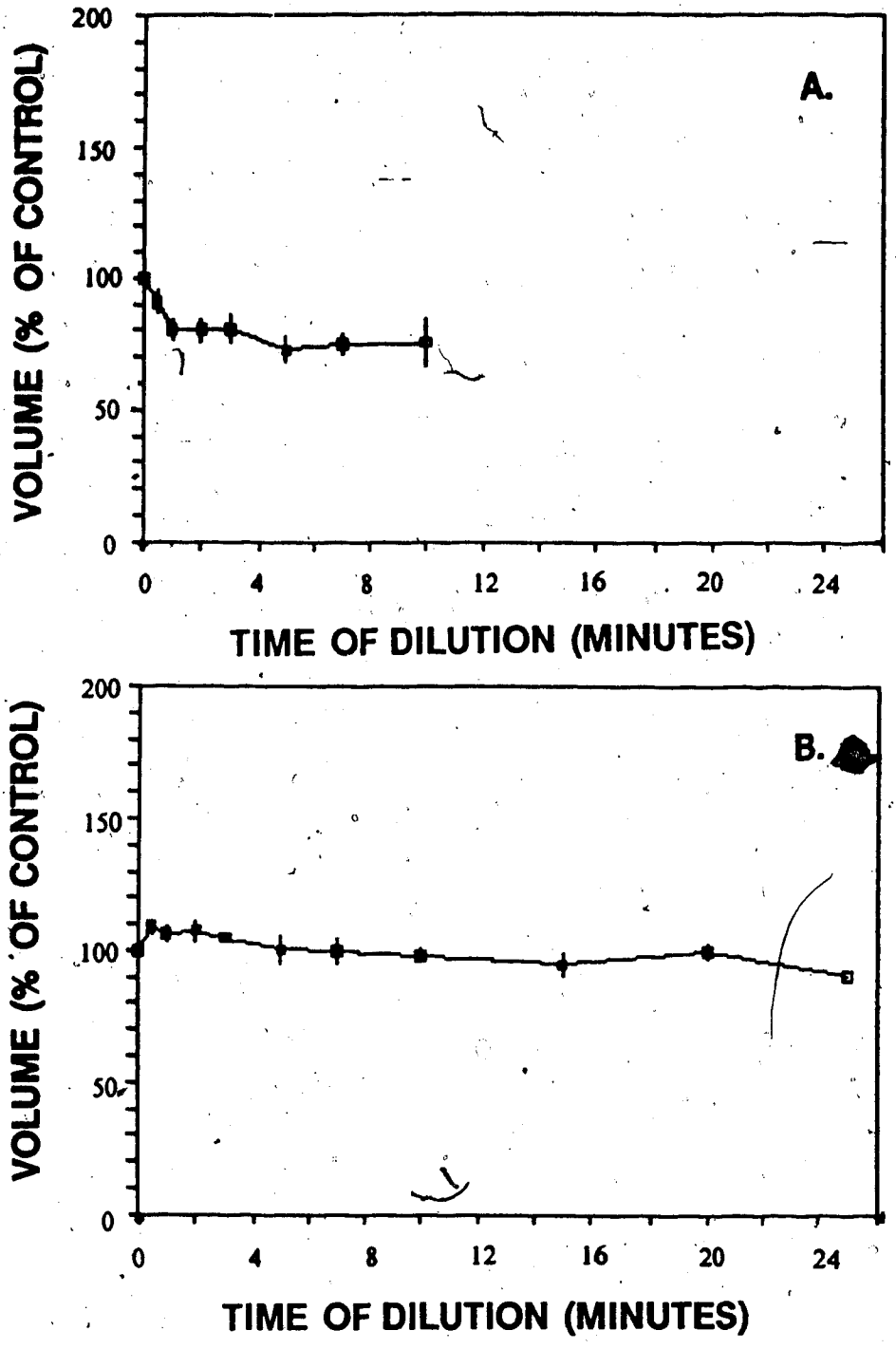

FIGURE 5.3.1 Change in lymphocyte volume, as a percent of the undiluted control, with increasing time of dilution in 0.1x medium and return to isotonic, at 25°C (A) and 0°C (B). Data are shown as mean +/- S.E.M, n=3.

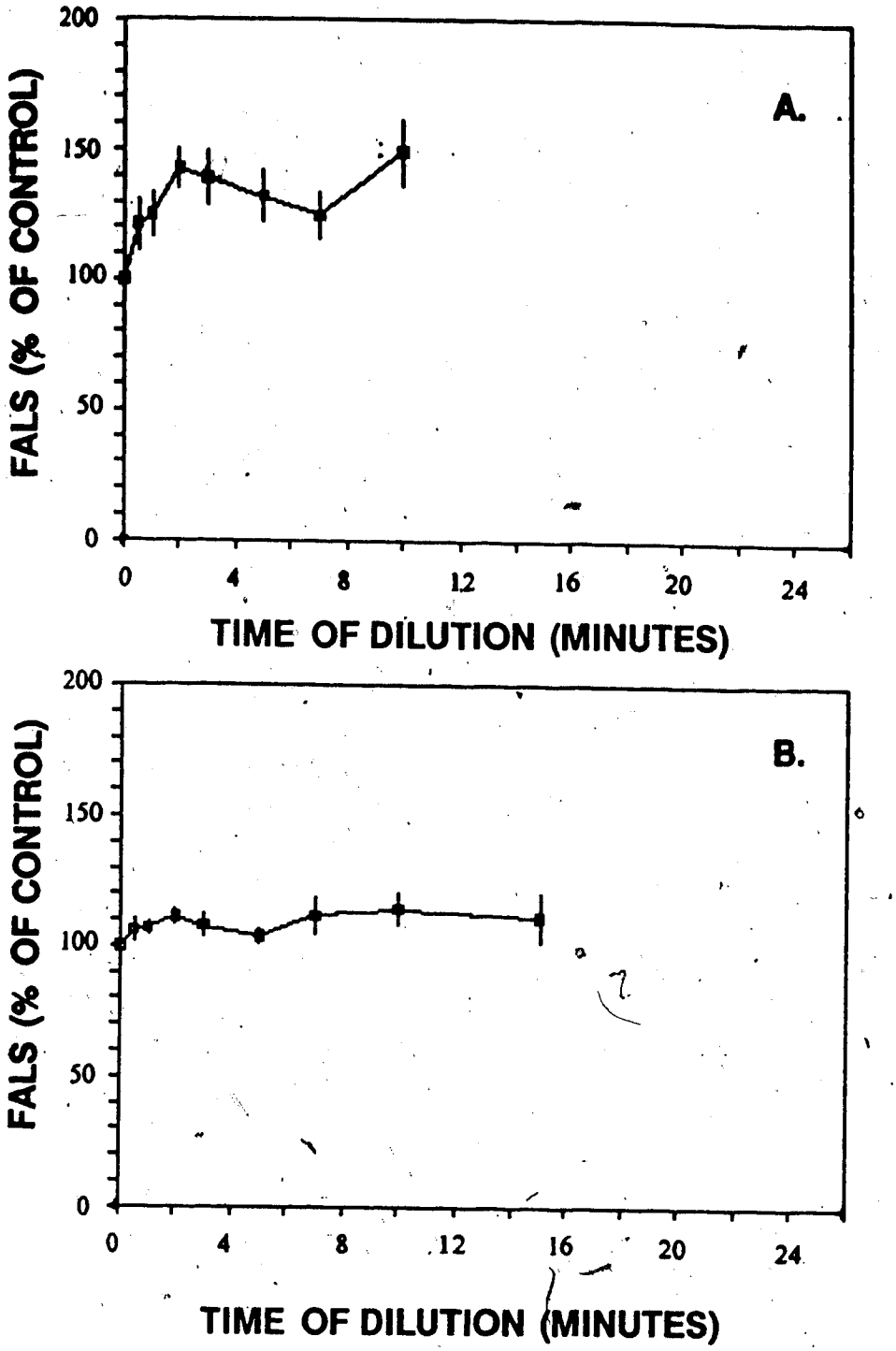

FIGURE 5.3.2 Change in lymphocyte FALS, as a percent of the undiluted control, with increasing time of dilution in 0.1x medium and return to isotonic, at 25°C (A) and 0°C (B). Data are shown as mean +/- S.E.M., n=3.

increasing duration of dilution at 25°C was observed (Fig. 5.3.3 A). The effect of dilution stress at 0°C on 90LS was less marked (Fig. 5.3.3 B).

Membrane potential-related fluorescence of surviving cells indicated depolarization in response to dilution stress at both experimental temperatures (Fig. 5.3.4).

5.3 (b) Increasing Dilution for Fixed Time, Return to Isotonic.

Changes in volume and FALS characteristics observed after exposure to stress and return to an isotonic environment are shown in Fig.'s 5.3.5 and 5.3.6. For both experimental temperatures, the volume and FALS curves are nearly mirror images of each other. When dilution was carried out at 25°C, changes were not evident in either parameter until the dilution exceeded 0.8x. Exposure to more dilute medium resulted in cell shrinkage (Fig. 5.3.5 a) and increased FALS intensity (Fig. 5.3.6 a). Dilution at 0°C, in contrast, produced little change in either parameter (Fig.s 5.3.5 b, 5.3.6 b).

90LS closely paralleled FALS (Fig. 5.3.7), but the observed increase in 90LS intensity at 25°C was less than the increase observed in FALS intensity at the same temperature.

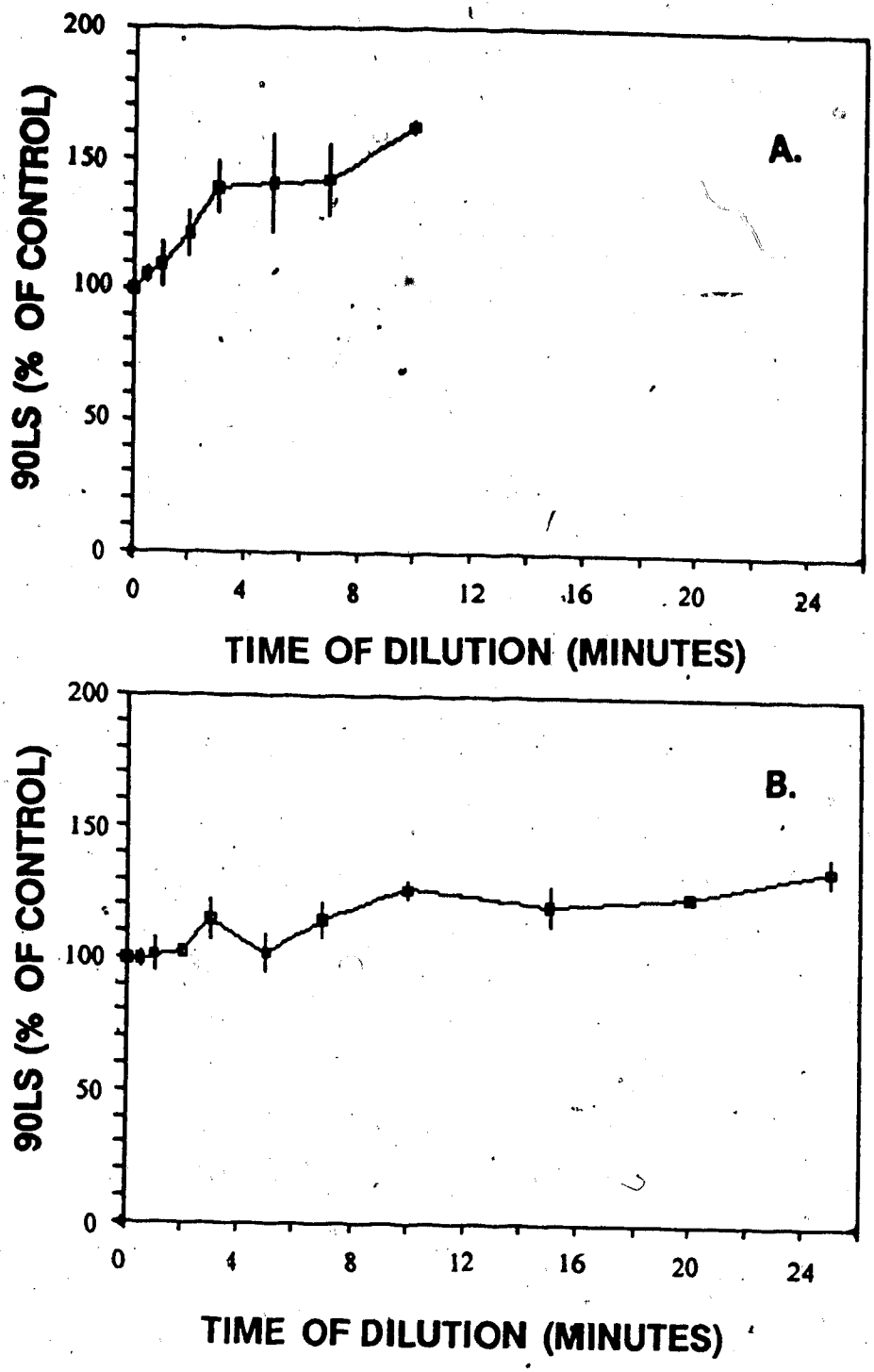

FIGURE 5.3.3 Change in lymphocyte 90LS intensity, as a percent of the undiluted control, with increasing time of dilution in 0.1x medium and return to isotonic at 25°C (A) and 0°C (B). Data are shown as mean +/- S.E.M., n=3.

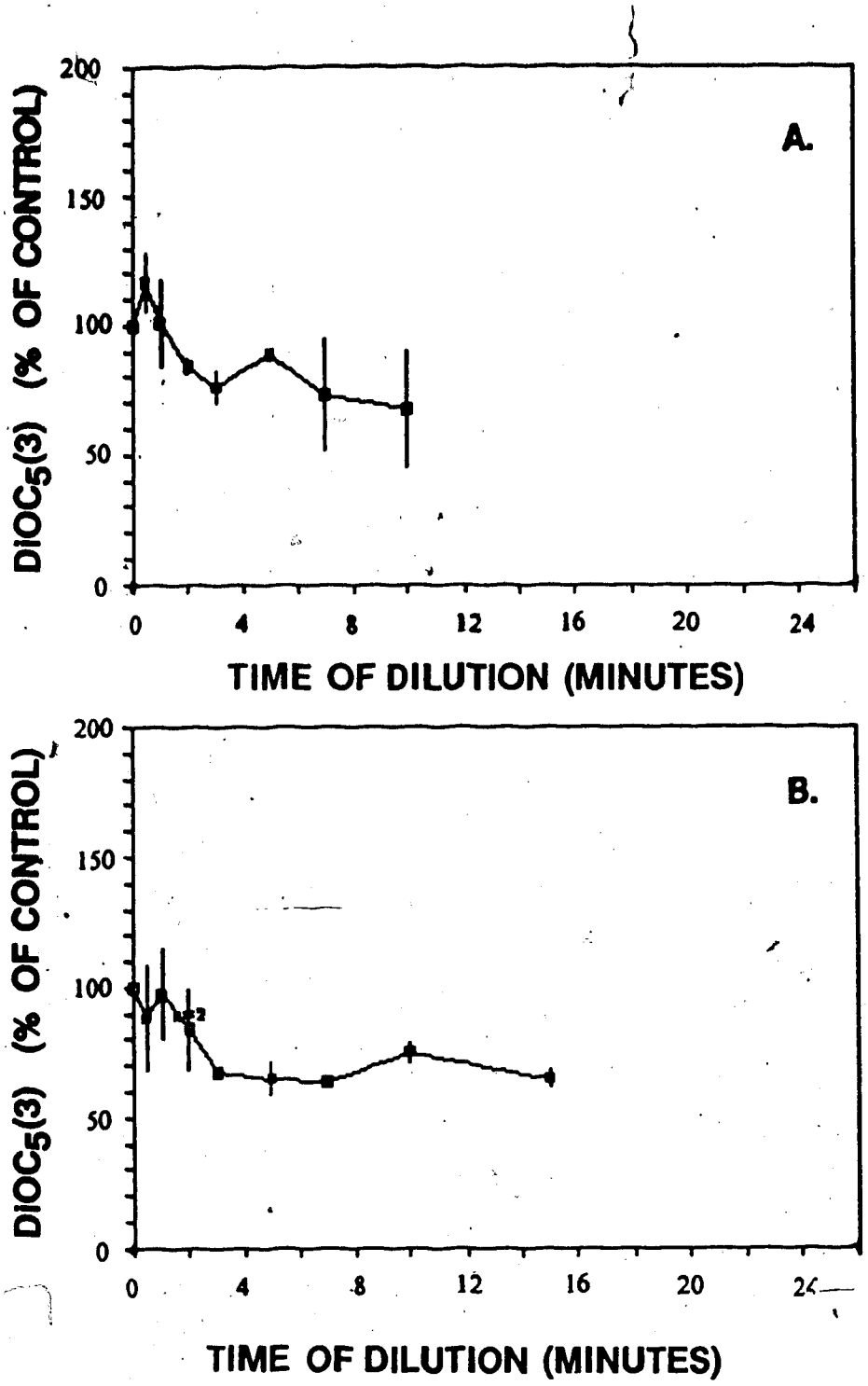

FIGURE 5.3.4 Change fluorescence intensity of $DiOC_5(3)$ expressed as percent of the undiluted control with increasing time of dilution in 0.1x medium and return to iostonic at 25°C (A) and 0°C (B). Data shown as mean +/- S.E.M., n=3.

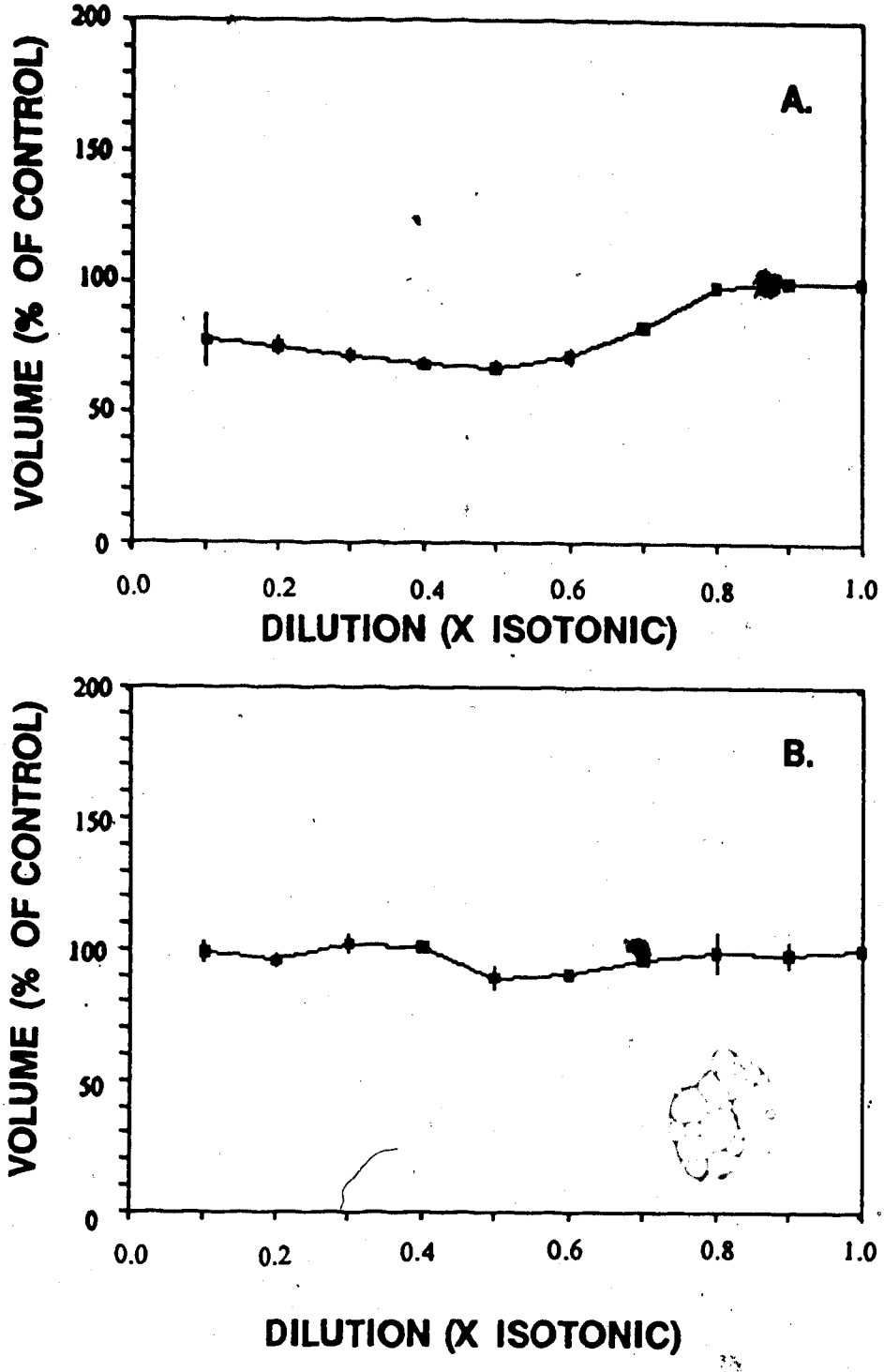

FIGURE 5.3.5 Change in lymphocyte volume, as a percent of the undiluted control, with increasing dilution for 5 min. and return to isotonic at 25°C (A) and 0°C (B). Data are shown as mean +/- S.E.M., n=3.

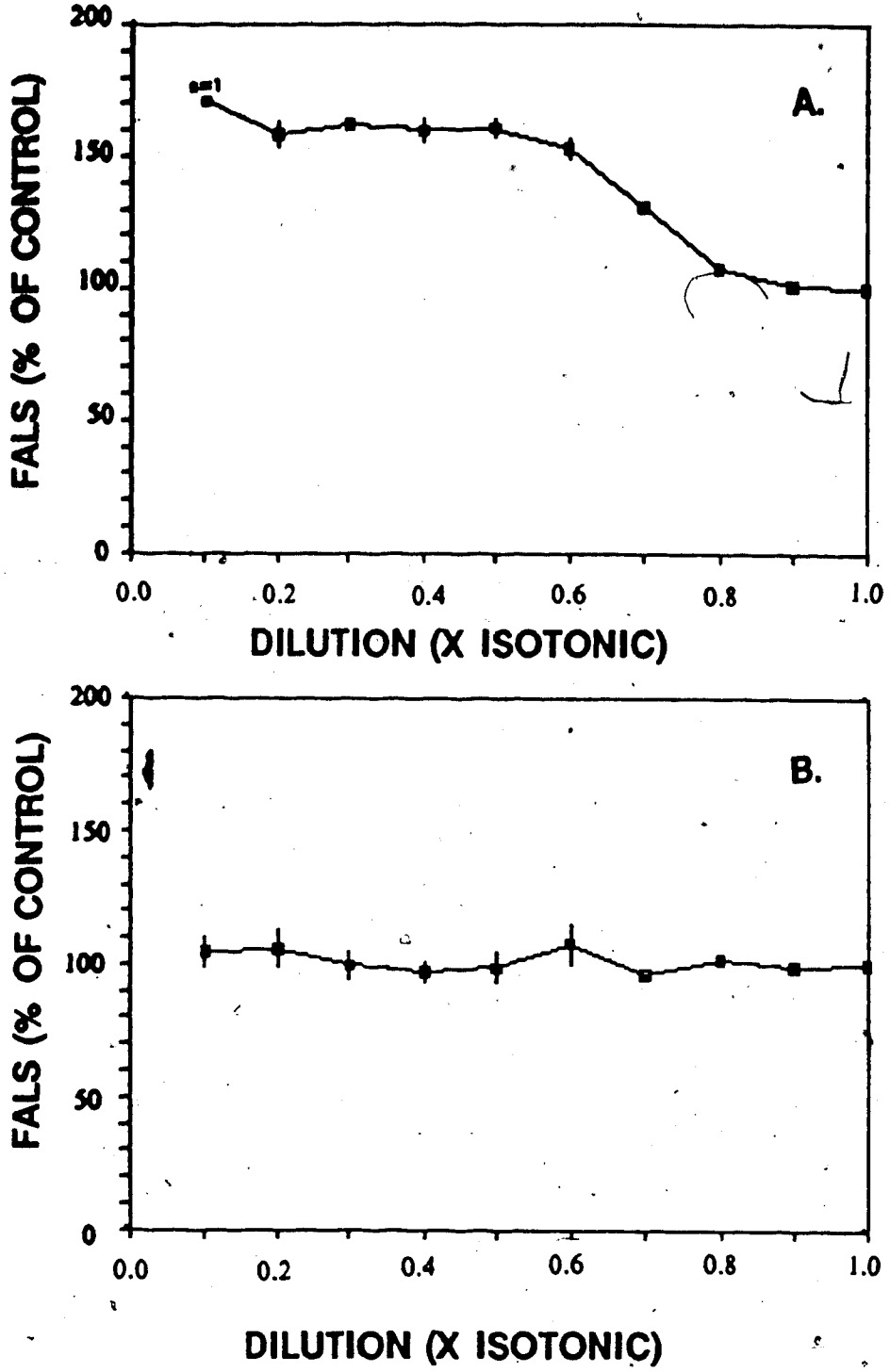

FIGURE 5.3.6 Change in FALS intensity, as a percent of the undiluted control, with increasing dilution for five minutes and return to isotonic, at 25°C (A) and 0°C (B). Data are shown as mean +/- S.E.M., n=3.

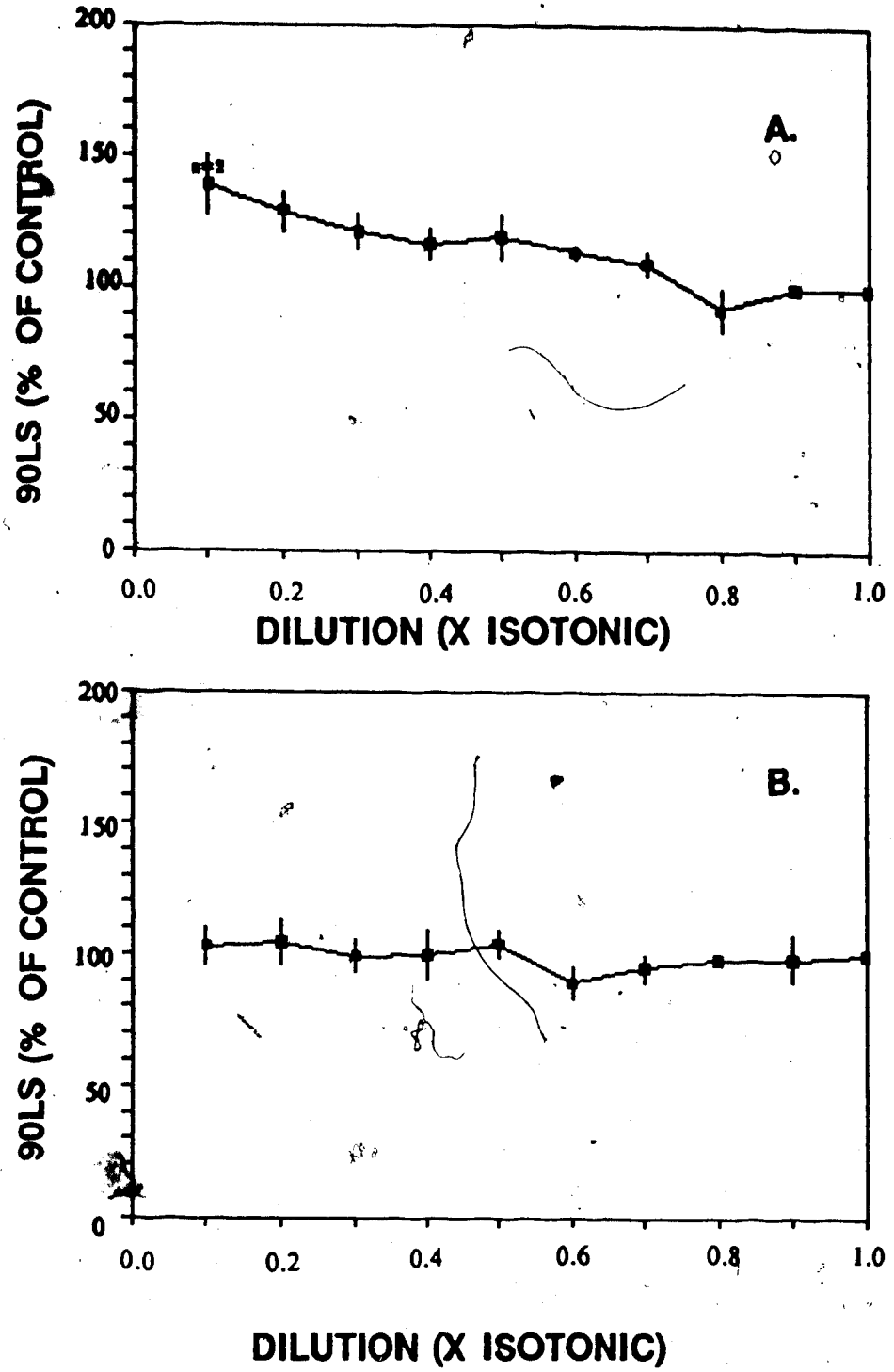

FIGURE 5.3.7 Change in 90LS intensity, as a percent of the undiluted control, with increasing dilution for five minutes and return to isotonic, at 25°C (A) and 0°C (B). Data are shown as mean +/- S.E.M., n=3.

A very gradual loss of membrane potential-related fluorescence was observed with increasing dilution stress when the cells were diluted at 25°C (Fig. 5.3.8 a). In samples diluted at 0°C, membrane potential fluorescence was very stable until dilution exceeded 0.5x; 0.4x, 0.3x, and 0.2x dilutions resulted in hyperpolarization (Fig. 5.3.8 b). Samples diluted in 0.1x medium at this temperature, however, were depolarized compared to the unstressed control.

5.4 VOLUME REGULATION AND ASSOCIATED CHANGES.

In order to investigate volume regulation, lymphocytes were exposed to a relatively mild dilution stress (0.6x). In this environment, at least 80% of lymphocytes retained membrane integrity at both experimental temperatures (Fig. 5.1.2).

Mild dilution at 25°C resulted in an immediate, rapid expansion of the peak volume to a maximum of approximately 130% of the isotonic volume within one minute (Fig. 5.4.1 a). This was immediately followed by a slower shrinkage phase, which resulted in the attainment of volumes within 5% of the isotonic volume by 6 min. after the onset of dilution.

When the dilution was carried out on ice, a rapid swelling phase was observed within the first 1-3 min.

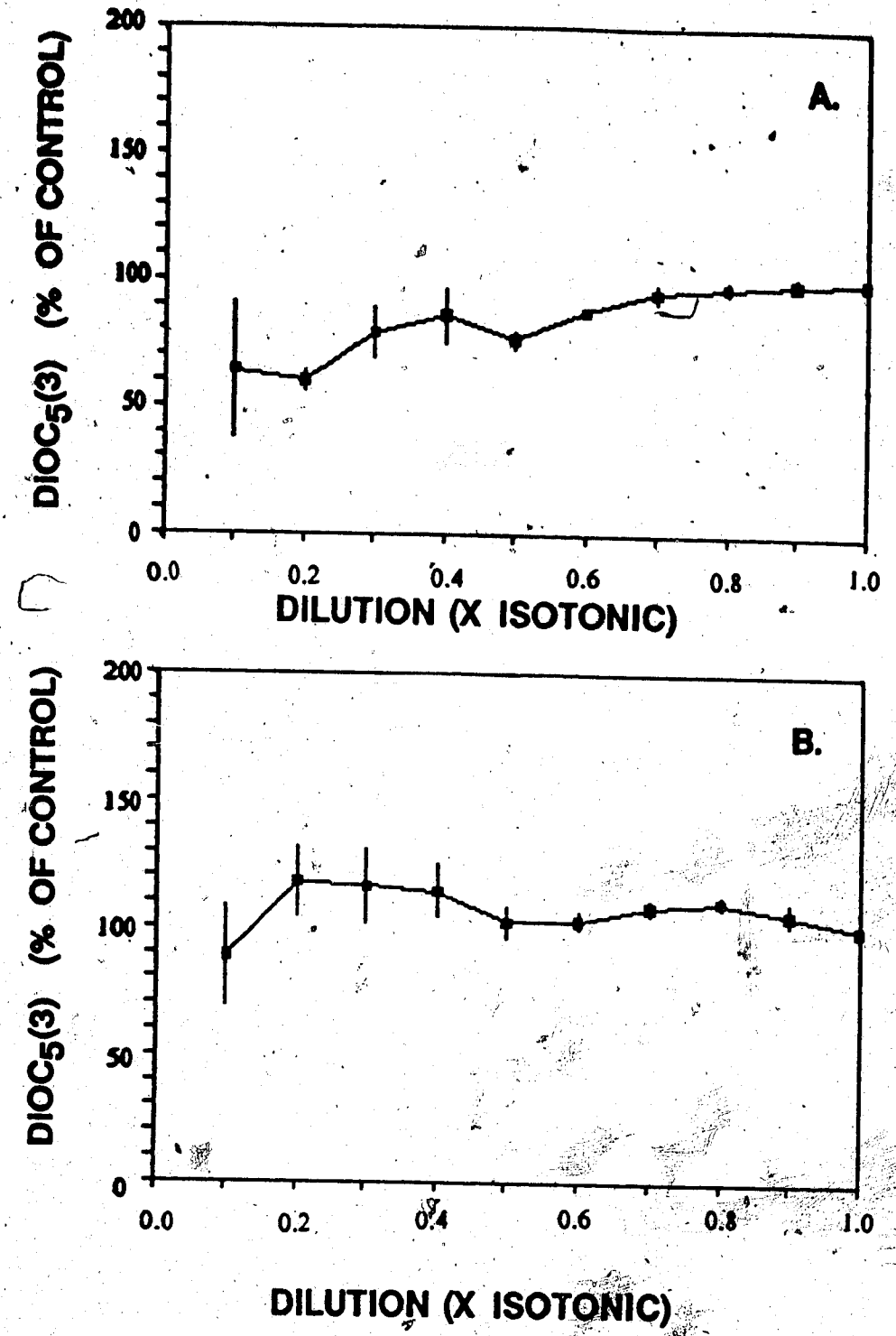

FIGURE 5.3.8 Change in fluorescence intensity of $DiOC_5(3)$ expressed as percent of the undiluted control, with increasing dilution for five minutes and return to isotonic, at 25°C (A) and 0°C (B). Data are shown as the mean +/- S.E.M., n=3.

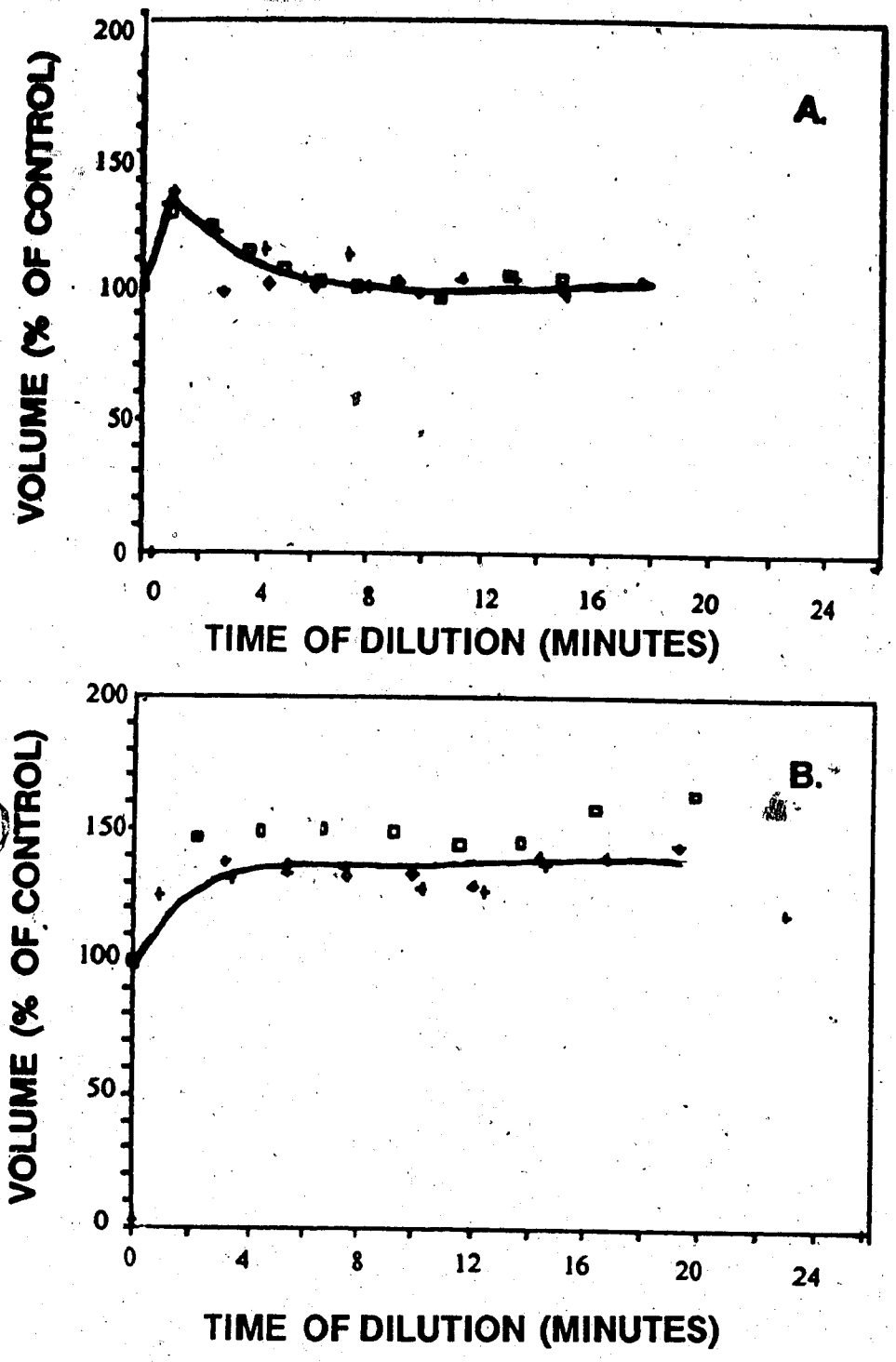

FIGURE 5.4.1 Change in lymphocyte volume, as percent of the undiluted control, with increasing time of dilution in 0.6x medium at 25°C (A) and 0°C (B). Individual data points are shown for three experiments. Curve drawn by hand.

after dilution, after which the lymphocytes remained swollen for the duration of the observation period (Fig. 5.4.1 b).

Fig. 5.4.2 shows that changes in FALS were approximately equal but in opposite direction to the changes in volume.

Two parameter histograms of volume versus FALS (Fig. 5.4.3) show graphically how the changes in these two parameters are related: Fig. 5.4.3 (A,B) shows that the increase in volume which occurred when cells were stressed at 25°C was coincident with the decrease in FALS (the shift in the distribution is both up and to the left in B compared to A). As the duration of stress increased beyond 3 min and cells began to shrink back towards isotonic volume, these same cells also regained FALS intensity (Fig. 5.4.3 C). The two parameter display, however, reveals a subpopulation of cells which remained expanded and with reduced FALS.

As in the previous stress protocol, changes in 90LS at both temperatures closely paralleled changes in FALS (Fig. 5.4.4).

The effect of mild dilution at 25°C on lymphocyte membrane potential was not investigated, because the membrane potential dye $DiOC_5(3)$ was observed to interfere with the volume regulatory response.

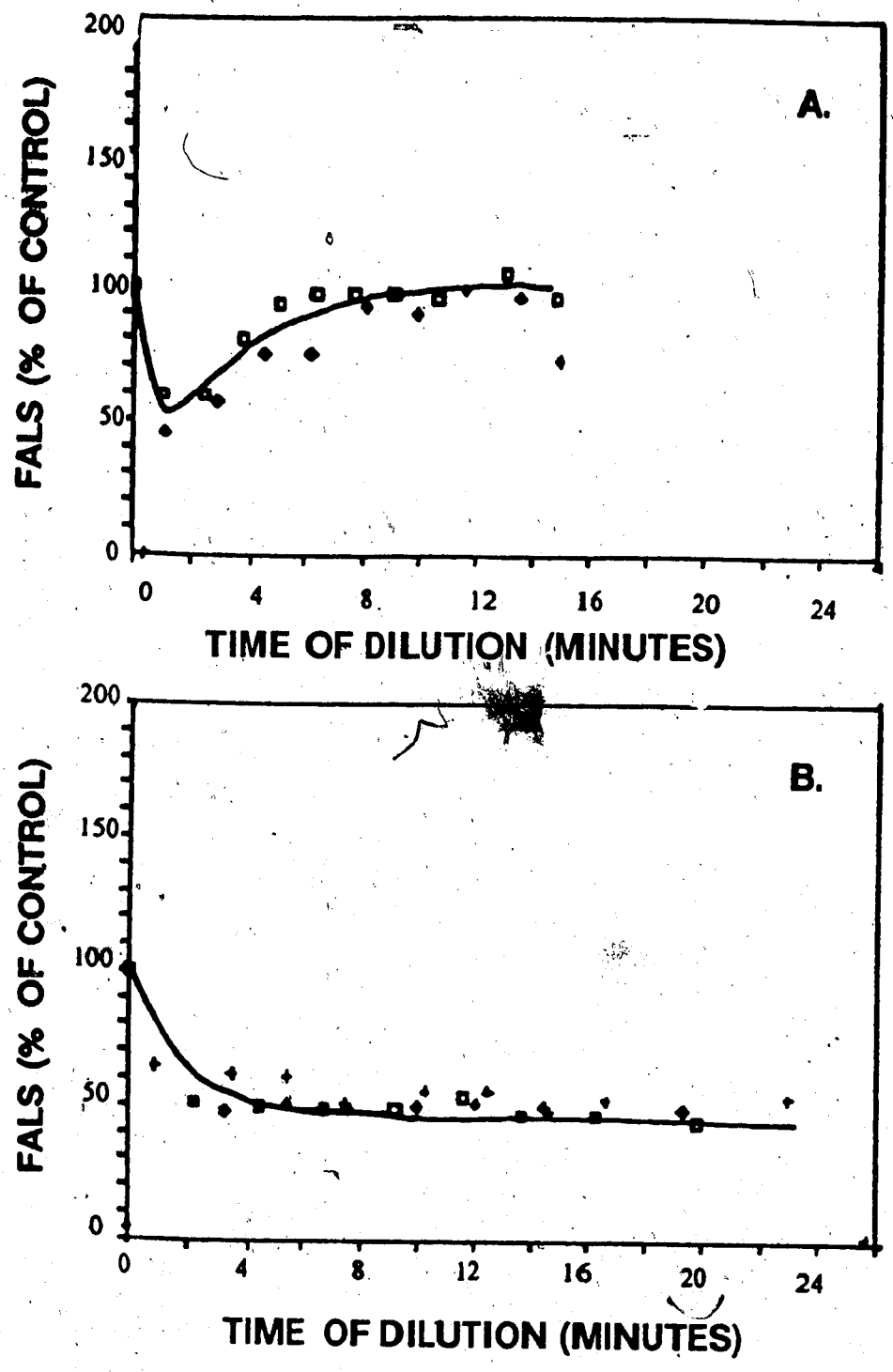

FIGURE 5.4.2 Change in lymphocyte FALS intensity as percent of the undiluted control, with increasing time of dilution in 0.6x medium at 25°C (A) and 0°C (B). Individual data points are shown for three experiments. Curve drawn by hand.

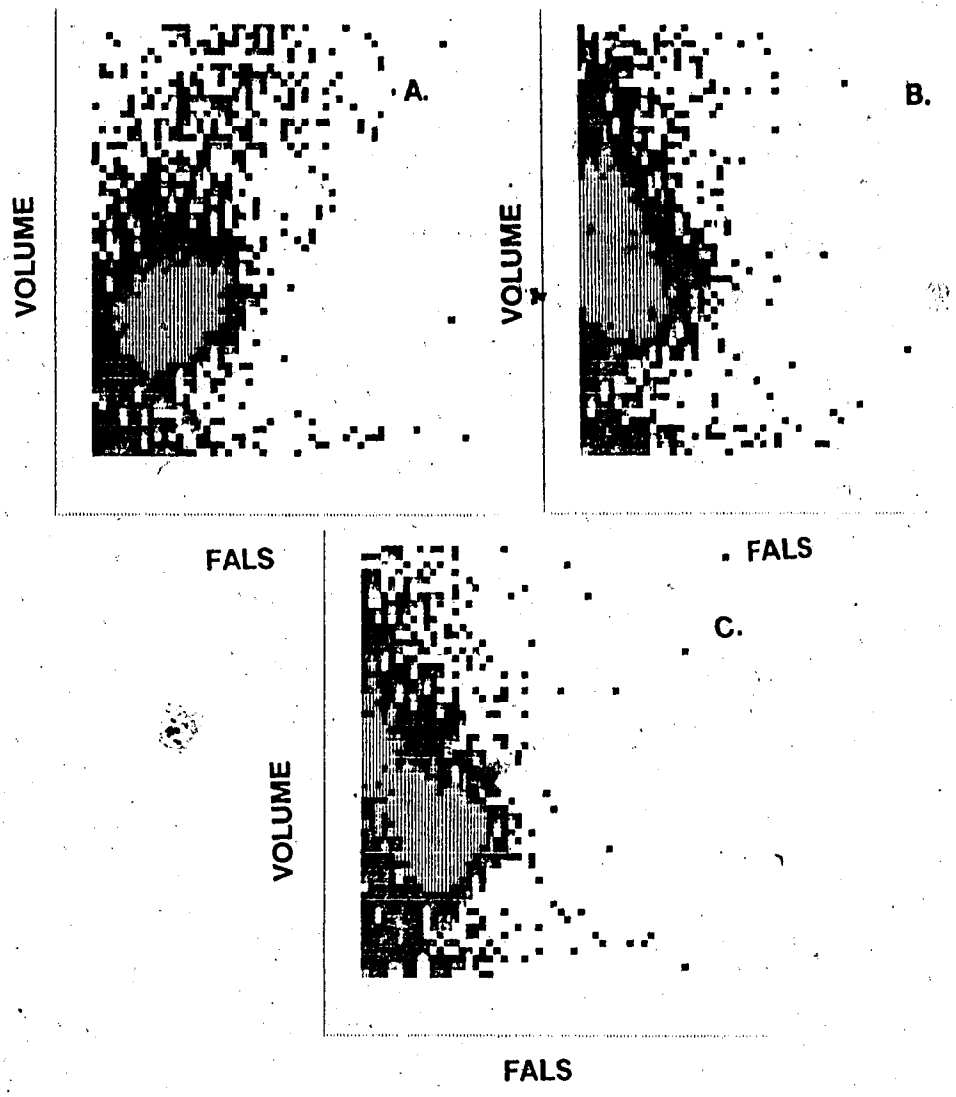

FIGURE 5.4.3 Two parameter histograms of FALS (horizontal axis) versus volume (vertical axis) for lymphocytes exposed to 0.6x dilution at 25°C for A) 0 min., B) 1 min., C) 7 min.

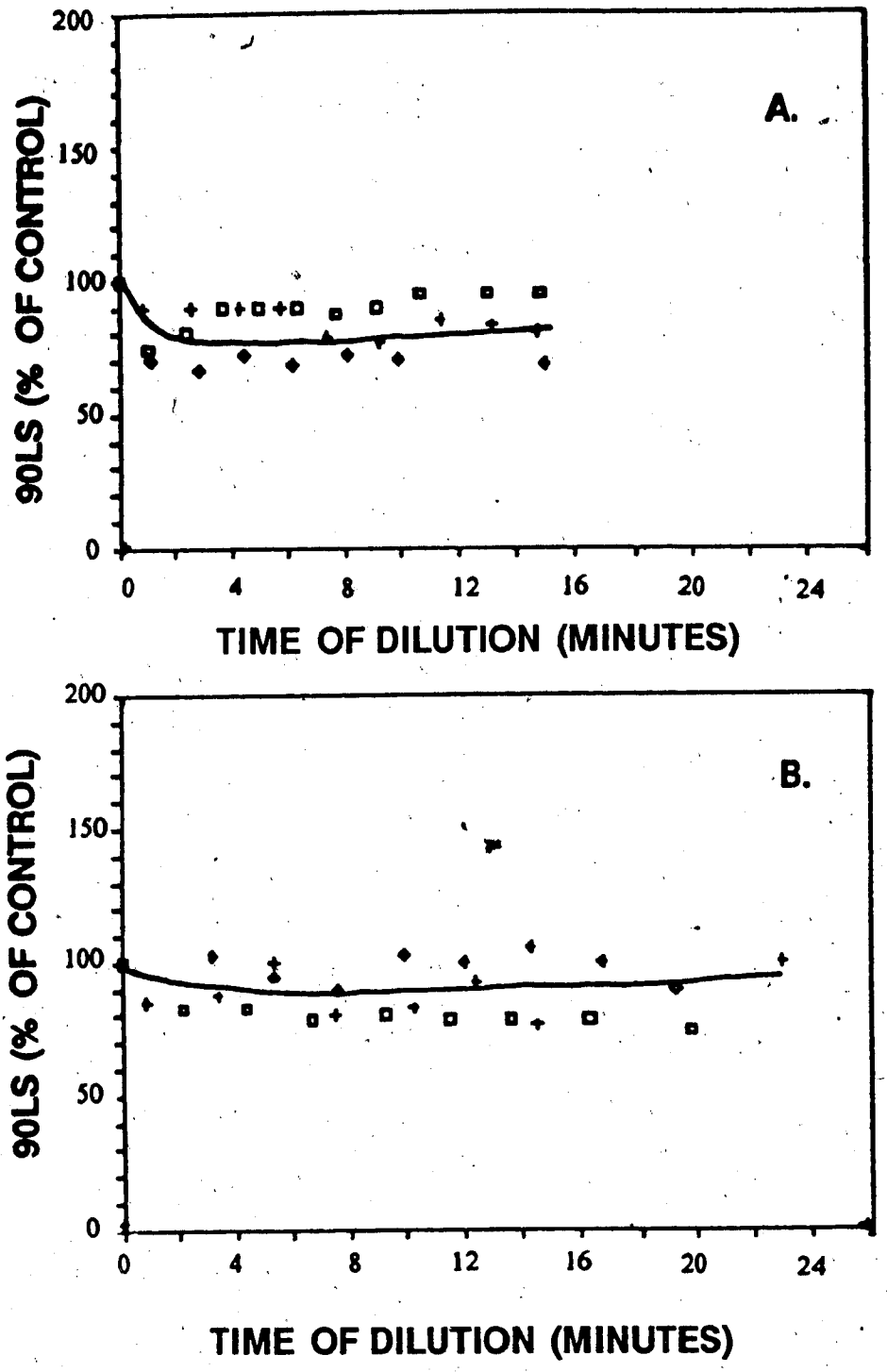

FIGURE 5.4.4 Change in 90LS intensity, as percent of the undiluted control, with increasing time of dilution in 0.6x medium at 25°C (A) and 0°C (B). Individual data points are shown for three experiments. Curve drawn by hand.

CHAPTER SIX

DISCUSSION

6.1 VARIABLES AFFECTING LYMPHOCYTE SURVIVAL IN A HYPOTONIC ENVIRONMENT.

The observation that mild dilution (0.5x - 0.3x) was more detrimental to cells at 0°C than at 25°C is in agreement with the results of Thorpe, Knight, and Farrant (1976), who found that removal of 10% DMSO by dilution in several volumes of isotonic medium with added serum was much more harmful to mouse lymphocytes when performed at 0°C than at 25°C, even in unfrozen cells. This work is the basis of the recommendation that post-thaw dilution of cryoprotectants be carried out at room temperature or above (Farrant, 1980).

The observation that extreme dilution (0.1x) was more detrimental to lymphocytes at 25°C than at 0°C suggests that the mechanism of cell damage may be different in the two tonicity ranges. Or, alternatively, a change in fluidity associated with a membrane lipid phase change could account for the difference observed between 0°C and 25°C.

It also suggests that the recommendation to carry out the post-thaw dilution step at room temperature is only practical if it is carried out slowly or in a stepwise manner, so as to minimize the dilution stress. If, however, as is very frequently the case, the dilution is

carried out rapidly in one step, it should be done on ice instead of at room temperature.

Thompson, Bull and Robinson (1966), in examining the pathogenesis of cell death as a result of exposure to hypotonic solutions, identified two distinct processes which led to cell death. Both occurred at the same temperature. One process occurred principally by membrane rupture with release of a relatively normal nucleus, whereas the other was characterized by pyknotic nuclear degeneration, with membrane rupture as a secondary event.

Schrek et al. (1980) described two distinct processes leading to cell death as a result of hyperthermia: "pyknotic death", in which the nucleus shrinks in size and the chromatin condenses, and "condensation." Pyknotic death occurred at temperatures between 37°C and 42°C, whereas condensation occurred above 42°C.

Two distinct morphological patterns of cell death in general have been identified, and have been found to occur under different circumstances (see review by Wyllie, et al., 1980).

In coagulative necrosis, a critical event takes place whereby cellular volume homeostasis is lost. Swelling precedes rupture of the plasma and organelle membranes and complete loss of organized structure.

Coagulative necrosis is characteristically the result of gross environmental perterbation by agents such as toxins, major changes in environmental temperature complement mediated lysis, and severe hypoxia. It is generally agreed that initial alterations in membrane permeability may be reversible, but that eventually increases in the intracellular levels of certain constituents such as Ca^{2+} may result in damage to the mitochondrial respiratory apparatus.

The other morphological pattern has been called shrinkage necrosis (Kerr, 1965; 1971) and apoptosis (Kerr et al., 1972). It is characterized by condensation of the cell with maintenance of organelle integrity. Surface protuberances, or "blebs" form and separate as membrane bound apoptotic bodies. The nuclear outline becomes abnormally convoluted and later, grossly indented. The cytoplasm condenses, and organelles are crowded together. Translucent cytoplasmic vacuoles are frequently associated with the overall cytoplasmic compaction.

Apoptosis is associated with focal deletion of cells during normal embryonic development and metamorphosis, and occurs spontaneously in growing neoplasms. It is also the pattern of death induced by radiation, cytotoxic cancer-chemotherapeutic drugs, and cell mediated immune responses.

6.2 CHANGES DETECTED IN LYMPHOCYTES ASSOCIATED WITH LOSS OF MEMBRANE INTEGRITY.

Flow cytometry has the potential to provide new information about the process of cell death. The sequence of changes in light scatter and fluorescence which are observed as cells die can be quantified for an entire population of cells.

Gross changes to cell organelles, such as pyknosis, nuclear convolution, or the appearance of cytoplasmic vacuoles would be expected to result in measurable changes in 90LS intensity. An increase in 90LS intensity might be observed in the early stages of nuclear condensation. Further condensation, if accompanied by shrinkage of the nucleus, might result in a secondary loss of 90LS intensity at the time of cell death.

Unfortunately, it was not possible to investigate the 90LS characteristics of dying cells in this study. An electrical noise problem was present in the 90LS PMT on the EPICS V flow cytometer. This noise was eliminated, however, when 90LS was gated on the FALS associated with surviving cells, allowing the observations in the next section (6.3) to be made.

The observation that as cells die, membrane potential is lost as FALS decreases is not surprising, since loss of FALS is known to be correlated with loss

of membrane integrity.

A decrease in FALS argues against the possibility of apoptosis as the mechanism of cell death as a consequence of hypotonic stress, since the development of surface blebbing associated with apoptosis would be expected to cause increased FALS.

The observation that volume is reduced secondary to loss of membrane integrity is at least partly due to the dependency of accurate electrical sizing on differences between the electrical resistivities inside and outside of the cell (Grover et al., 1982a).

6.3 CHANGES IN FLOW CYTOMETRIC PARAMETERS DETECTED IN SURVIVING CELLS.

The effects of hypotonic stress were described for cells which retained membrane integrity. A common feature of both stress protocols was that surviving cells had reduced volume and increased FALS intensity when stressed at 25°C, but not when stressed at 0°C.

FALS and volume change in opposite directions in response to movement of water both out of cells (McGann, Walterson and Hogg, 1987) and into cells, as shown in this study. Expansion of cell volume could result in a decrease in the amount of light scattered either by changing the refractive index of the cell, or by smoothing surface projections. 90LS is not helpful in distinguishing these two possibilities, since changes in

90LS paralleled changes in FALS.

Loss of volume indicates that during exposure to a hypotonic environment, lymphocytes lost intracellular ions. When returned to isotonic conditions, more water was obliged to leave the cell than entered during osmotic swelling in order for osmotic equilibrium to be reached.

Whether loss of ions was due to a generalized loss of selective permeability or due to changes in permeabilities of specific ions is difficult to ascertain. Evidence for a change in permeability to specific ions, as would occur in a volume regulatory response, is found in the observation that loss of volume was not observed in lymphocytes stressed at 0°C. Lymphocyte volume regulation does not occur at 0°C (Grinstein et al., 1984).

The observation that dilution in 0.8x medium constituted a threshold for volume and light scatter changes after stress also supports specific changes in permeability associated with the volume regulatory response. When exposed to a solution of this osmolality, lymphocytes initially swell to 1.19x their isotonic volume (Law et al., 1983). The volume expansion threshold to trigger the opening of the Cl^- ion pathway is 1.15x the isotonic volume (Sarkadi et al., 1984a). In 0.9x medium, the chloride pathway is

not open, and cells do not experience increased permeability to Cl^- ions during a five minute exposure to this medium.

In addition to being reduced in volume, cells also became increasingly depolarized with increased duration of exposure to severe (0.1x) dilution at both experimental temperatures, and with increasing severity of dilution beyond 0.7x at 25°C. Depolarization was not evident in cells exposed to solutions less dilute than 0.2x at 0°C.

Depolarization could result from an increase in Na^+ permeability, or from a loss of intracellular K^+ and resulting reduction in the K^+ equilibrium potential. Alternatively, depolarization might indicate a generalized loss of selective permeability. This early depolarization, preceeding loss of FALS, might be indicative of early and reversible damage.

6.4 LYMPHOCYTE VOLUME REGULATION DURING MILD DILUTION

The observation that lymphocytes regulate their volumes during mild dilution at 25°C has been made by others (Ben-Sasson et al., 1975; Bui and Wiley, 1981; and Cheung et al., 1982). Use of a flow cytometer to investigate this phenomenon allowed simultaneous measurements to be made of volume and light scatter. Changes in light scatter corresponding to volume changes are evident for light scattered in both forward and

orthogonal (90°) directions. This means that volume regulating and non-volume regulating cells can be distinguished by up to three intrinsic parameters using a flow cytometer.

This study confirms the observations of Cheung et al. (1982) that a subpopulation of cells remains swollen during volume regulation in hypotonic medium. By examining the volume responses of pure populations of B lymphocytes, Cheung et al. found that B cells do not exhibit a volume regulatory response.

It is possible that differences between subpopulations in ability to regulate volume in a hypotonic environment may be correlated with differences in susceptibility to hypotonic stress. Murine B cells are more resistant to hypotonic damage than T cells (Strong et al., 1975). Human B cells do not exhibit volume regulation (Cheung et al, 1982). Lymphocytes from leukemic patients have been found to have diminished volume regulation compared to normal lymphocytes (Ben-Sasson et al., 1975), and have been found by several investigators to be more resistant than normal cells to hypotonic stress (Westring and Britten, 1968; Thompson, Robinson and Wetherly-Mein, 1966, and Storti and Pederzini, 1956).

That the two properties might be functionally related in some way suggests that interfering with the

capacity of a cell to regulate its volume in hypotonic medium might confer increased resistance to hyptonic stress, and warrants further investigation.

The physiological significance of volume regulation in lymphocytes remains obscure, since the deviations from isoosmolality *in vivo* are small compared to the range of conditions under which lymphocytes are able to maintain volume *in vitro* (Pollock and Arieff, 1980).

It is possible that there is no functional significance to volume regulation. Changes in K^+ permeability and mediation by Ca^{2+} are common to both volume regulation and lymphocyte activation in response to antigens. Physical changes in the lymphocyte membrane occurring during swelling might mimic early membrane changes during antigenic stimulation, leading to increased K^+ permeability. In a hypotonic environment, the consequence is loss of volume.

The observations that different cell types vary in the extent to which they regulate their volumes when exposed to mild dilution, and that the volume regulatory response is affected by temperature, have important implications for cell separation technology. Exposure to a mild dilution could, for instance, allow elutriation by counterflow centrifugation of cells which are otherwise indistinguishable by volume alone.

CHAPTER SEVEN

CONCLUSION

The effects of exposure to a hypotonic environment on human lymphocytes were characterized by flow cytometry. There were four areas of focus: factors effecting lymphocyte survival, changes associated with cell death, changes associated with cell survival, and lymphocyte volume regulation.

Exposure to severe dilution was found to be more detrimental to lymphocyte survival at 25°C than at 0°C. Exposure to less dilute solutions, however, was more detrimental at the lower temperature, suggesting that two different mechanisms of cell death may be involved.

Cell death was characterized by loss of FALS intensity accompanied by loss of membrane potential. Loss of cell volume was observed as a late event.

Lymphocytes which survived a dilution stress showed evidence of loss of intracellular ions. Two hypotheses to explain this observation are proposed: that ions are lost during volume regulation, or that ions are lost as a result of early, perhaps reversible, loss of selective permeability. Evidence presented here favours the former hypothesis.

Lymphocytes were observed to regulate their volumes in hypotonic medium at 25°C, but not at 0°C. At the higher temperature, a subpopulation was observed

which did not regulate volume. It is suggested that a relationship may exist between the ability of cells to regulate volume in a hypotonic environment and susceptibility to hypotonic stress.

In conclusion, hypotonic stress is not a simple environmental perturbation which either leads to cell lysis or leaves cells unchanged. As these results show, hyptonic stress exerts compound influences on lymphocytes which are different at different temperatures, which may be passive or physiological, and which selectively effect certain subpopulations of cells. Flow cytometry provides a useful tool with which to examine the sequence of events which occur when cells are challenged by alterations to the environment.

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APPENDIX A

DETAILS OF DILUTION AND CORRECTION SOLUTIONS USED IN PROTOCOL 4.2b.

The protocol for preparation of solutions of variable tonicity (30 - 300 mOsm/kg), and of the appropriate solutions for correction to isotonic (300 mOsm/kg) is presented in Table I.

TABLE I. Protocol for dilution of cells in media of variable hypotonicity and correction to isotonic.

10 x PBS (µl)	Distilled of Water (µl)	Tonicity of Dilution Medium (x isotonic)
90	810	1
80	820	0.9
70	830	0.8
60	840	0.7
50	850	0.6
40	860	0.5
30	870	0.4
20	880	0.3
10	890	0.2
0	900	0.1

To each 900 ul of medium was added 100 µl of cell supension in 1x medium at 20 x 10^6 cells/ml.

100µl of the appropriate hypertonic medium necessary to correct the tonicity are added to 1 ml of the hypotonic cell suspension. The final tonicity is isotonic. For example: the sample which was originally 0.6x, upon addition of 100 µl of 5x solution, consists of 50 + 10 + 50 = 110 µl of 10x PBS in a total volume of 1100 ul.

APPENDIX B

FLOW CYTOMETER SPECIFICATIONS

The following are the specifications for laser power, laser wavelength, filters, gain, and PMT voltage used in this study.

1. Laser Voltage The laser power was maintained at 500 mW for all experiments.

2. Laser Wavelength The wavelength of coherent light emitted by the laser was 488 nm.

3. Filter Setup The filter setup used in this study was as follows: 488 nm dichroic filter at 45° to the FALS detector, 457-502 nm laser blocking filter at 90° to the path of light, 550 nm long pass dichroic at 45° to the green fluorescence detector (used for membrane potential related fluorescence), and a 525 nm bandpass filter directly in front of the green detector.

4. Gain and High Voltage Settings Details of the gain and high voltage settings used for each PMT are presented in Table II.

TABLE II. Gain and high voltage settings applied to each detector.

Detector	Parameter	Gain	High Voltage (mW)
PMT1	FALS	10	-
PMT3	green fluorescence (membrane potential)	20	700-800
PMT4	90LS	-	650
CVA	volume	5	-

APPENDIX C

MEMBRANE POTENTIAL CALIBRATION

$DiOC_5(3)$ fluorescence was tested for response to membrane potential by exposing lymphocytes to varying extracellular concentrations of K^+ and by the use of valinomycin, a K^+ ionophore.

1. Methods

Isotonic solutions of PBS and KCl with phosphate buffer were prepared. The molar constiuents of both are listed in Table III. The osmolarity of both solutions was determined, using a freezing point depression osmometer (Precision Systems Osmette), to be 290 +/- 4 mOsm.

TABLE III: Composition of Isotonic PBS and KCl Solutions

Solution:	1x PBS (mMolar concentration)	1x KCl (mMolar concentration)
NaCl	136.9	8.0
$Na_2PO_4.7H_2O$	8.1	8.1
Total Na^+	153.1	24.2
KCl	2.7	155.0
KH_2PO_4	1.5	0
Total K^+	4.2	155.0

The two solutions were mixed in the relative proportions 100/0, 75/25, 50/50, 25/75, and 0/100, to yield isotonic solutions with concentrations of ionized Na^+ and K^+ as shown in Table IV.

TABLE IV: Concentrations of Na+ and K^+ in solutions used in the calibration of membrane potential.

Proportion PBS/KCl	Na^+	K^+
100/0	153.1	4.2
75/25	120.9	42.0
50/50	88.7	79.6
25/75	56.5	127.3
0/100	24.2	155.0

Lymphocytes were incubated in the above solutions in the presence of 100nM $DiOC_5(3)$ for 30 min. in the dark at room temperature.

Valinomycin was prepared in DMSO and added to achieve a final concentration of 10 μM.

Fluorescence data was collected using linear integrated green fluorescence gated on FALS and volume.

2. Results

Depolarizing cells by increasing extracellular K^+ while keeping the total osmolality of the solution constant resulted in decreased fluorescence intensity

(Fig. App.C). Valinomycin produced increased fluorescence in low K^+ medium. The extent of the increase in fluorescence intensity produced by valinomycin decreased as the extracellular K^+ concentration increased.

These results indicate that Di-O-C_5(3) reports membrane potential at the dye and cell concentrations used in these experiments.

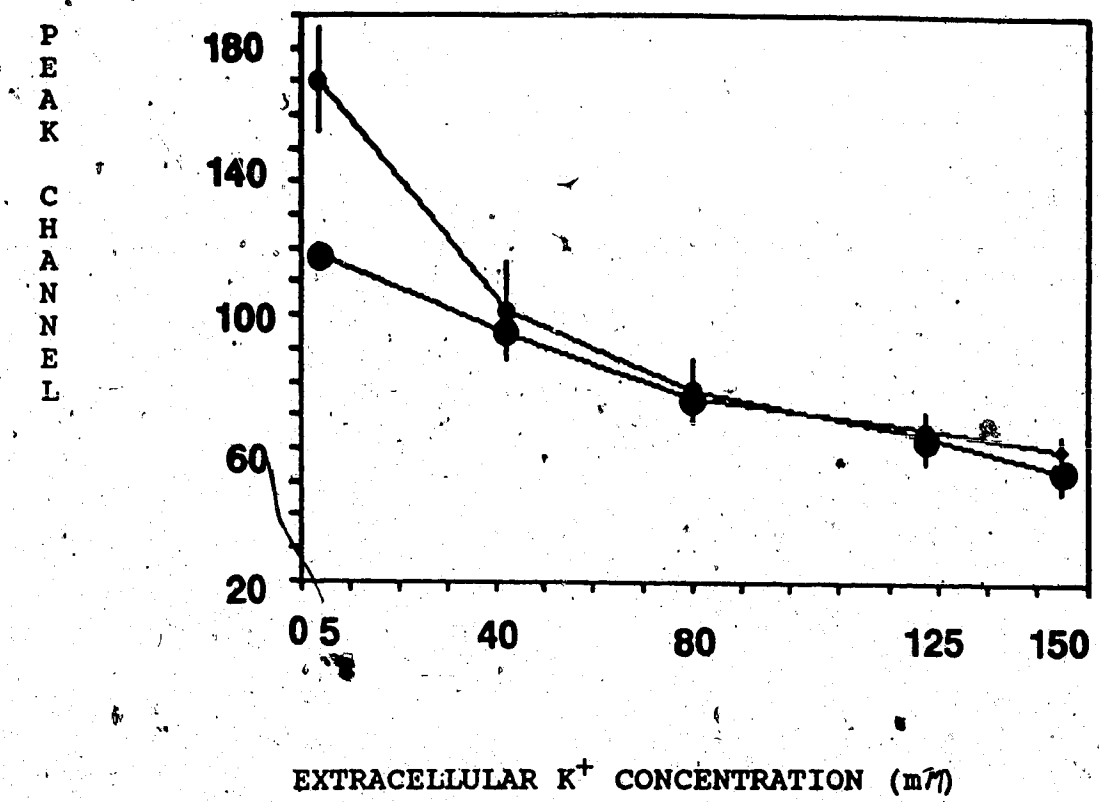

FIGURE APP.C Fluorescence intensity of $DiOC_5(3)$-labelled lymphocytes versus extracellular potassium ion concentration. Data are shown as mean +/- S.E.M., n=3. ● control, ● with valinomycin.

APPENDIX D

VOLUME AND FALS CHARACTERISTICS OF LIVE AND DEAD CELLS

Fluorescein diacetate (FDA) and ethidium bromide (EB) were used as indicators of membrane integrity (Rotman and Papermaster, 1966). FDA is a non-fluorescent, lipophilic compound which traverses the plasma membrane easily. Once inside the cell, the electroneutral esters are rapidly hydrolysed by esterases. The negatively charged fluorescein molecule is highly fluorescent, anionic, and does not readily cross the cell membrane. As long as the membrane is intact, fluorescein leaves the cell only very slowly in comparison to the rate of entry of FDA. Fluorescein fluorescence is therefore indicative of both membrane integrity and enzyme activity.

Ethidium bromide, in contrast, does not penetrate intact membranes, and is used to demonstrate loss of membrane integrity. Once inside the cell, it complexes with double stranded nucleic acids by intercalating between base pairs (LePecq and Paoletti, 1967).

1. Methods

Ethidium bromide, EB (2,7-diamino-10-ethyl-9-phenyl phenanthridium; Sigma) was prepared as a 2.5×10^{-4} M stock solution in ethanol and stored in the dark at 4°C. This stock solution was diluted 1/100, v/v, with PBS to make a working solution. The final concentration of EB

in the cell suspension was 25 µm.

Fluorescein diacetate (Molecular Probes) was prepared as a 1 x 10^{-4} M stock solution in acetone and stored at -10°C. The working solution was prepared as for EB, and the final concentration of FDA in the cell suspension was 10 µM.

2. Results (Fig. App. D)

Positive FDA fluorescence in unstressed samples was associated with high intensity FALS.

Positive EB fluorescence, in contrast, was associated with reduced FALS. Cell volumes in EB+ populations were more widely distributed than in FDA+ populations.

The results in Fig. App.D. illustrate how a gating window could be defined, based on FALS, to discriminate between live and dead cells.

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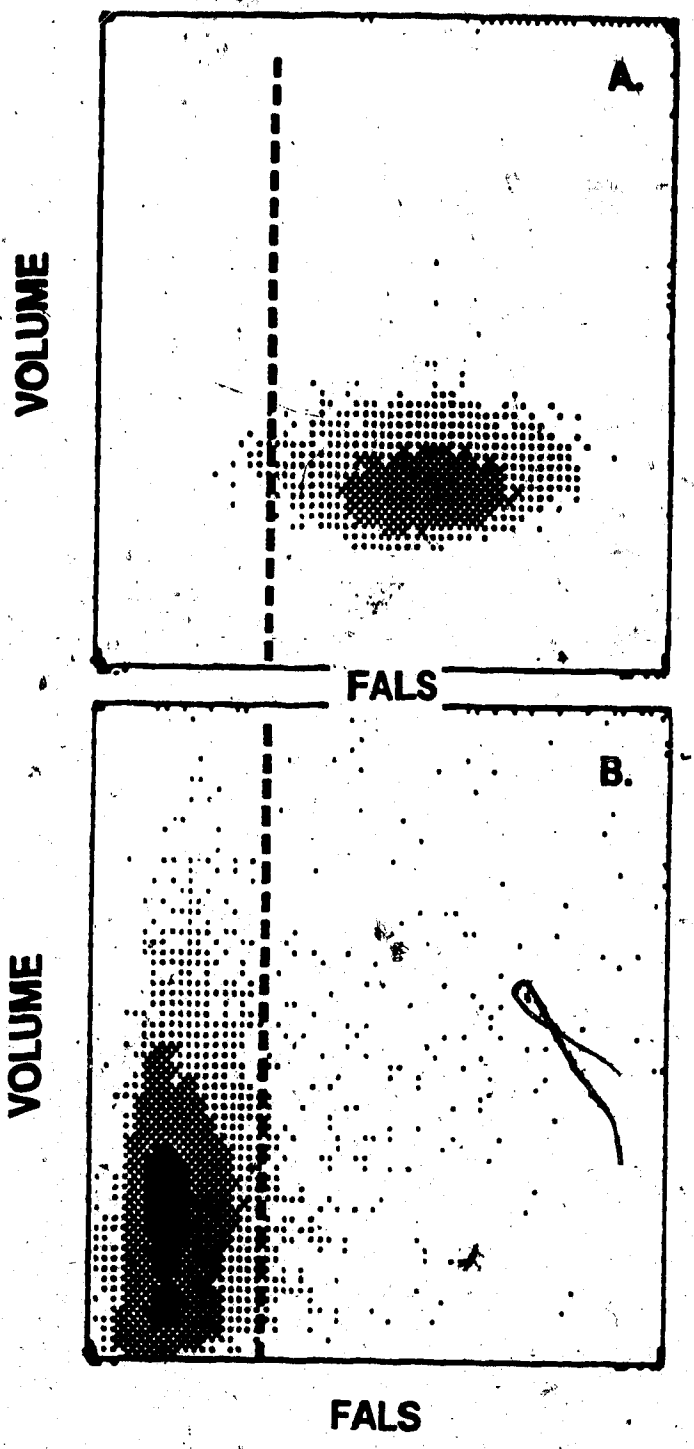

FIGURE APP.D Two parameter histograms of FALS (horizontal axis) versus volume (vertical axis) for lymphocytes exposed to 0.4x medium for 5 min. at 25°C . A) Gated on positive FDA fluorescence. B) Gated on positive EB fluorescence. The dotted line indicates where a FALS gate would be placed to exclude dead cells.