1983

Effect Of Hypotonic Media On Ehrlich Ascites Tumor Cells: Correlation Of Morphologic Changes With Ion And Water Movements.

Elizabeth Haughie Wilkerson
University of Alabama at Birmingham

INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.

2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of "sectioning" the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.

University Microfilms International
300 N. Zeeb Road
Ann Arbor, MI 48106

Wilkerson, Elizabeth Haughie

EFFECT OF HYPOTONIC MEDIA ON EHRLICH ASCITES TUMOR CELLS: CORRELATION OF MORPHOLOGIC CHANGES WITH ION AND WATER MOVEMENTS

The University of Alabama in Birmingham PH.D. 1983

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __√__.

1. Glossy photographs or pages ____✓____
2. Colored illustrations, paper or print _____
3. Photographs with dark background ____✓____
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages _____
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Other _____

Effect of Hypotonic Media on Ehrlich Ascites Tumor Cells: Correlation of Morphologic Changes with Ion and Water Movements

By

ELIZABETH HAUGHIE WILKERSON

A DISSERTATION

Submitted in partial fullment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology and Biophysics in The Graduate School,
The University of Alabama in Birmingham

BIRMINGHAM, ALABAMA

1983

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA IN BIRMINGHAM

Degree Doctor of Philosophy Major Subject Physiology and Biophysics

Name of Candidate Elizabeth Haughie Wilkerson

Title Effect of Hypotonic Media on Ehrlich Ascites Tumor Cells: Correlation of Morphologic Changes with Ion and Water Movements

Volume regulatory decrease, the reduction of cell volume by the extrusion of intracellular osmotically active solute, was studied in Ehrlich ascites tumor cells by correlating morphologic changes with ion and water movements during hypotonic stress.

When cell suspensions were diluted with distilled water to an osmolality one-third of isosmotic, they swelled within twenty-five seconds to 2.7 times their initial volume, behaving as nearly perfect osmometers. Both Na^+ and K^+ were diluted proportionally.

During the initial rapid swelling, numerous vesicle-like protrusions (blebs) formed instantaneously over most of the plasma membrane. The bleb contents appeared less dense than the remainder of the cell cytoplasm by ordinary light microscopy and with Nomarski optics. By scanning electron microscopy, the blebs appeared to be "ballooned" microvilli. With transmission electron microscopy, the bleb external membrane was seen to be continuous with the plasma membrane, and the bleb contents were less granular than the rest of the cytoplasm, but no definable structure separated the bleb from the remainder of the cell. Additionally, the bleb membrane contained antigenic markers, indicating it was probably preformed plasma membrane.

Nomarski optics was conjoined with computer based digitization of video images for morphometric studies. By tracing cell outlines before and during hypotonic stress, the increase in cell volume was found to be totally accounted for by the bleb volume.

For ten to twenty minutes after dilution, cell Na^+ content increased and K^+ content decreased. Cell volume increased initially, but subsequently decreased by approximately twenty-seven percent of its maximum. Later, Na^+ decreased and K^+ increased, moving against their chemical gradients, and volume decreased an additional eleven percent. Amiloride, ouabain, colchicine, or dibutyryl cAMP had no effects on the volume, Na^+, or K^+ changes. Furosemide appeared to decrease the magnitude of the Na^+ entry and to increase slightly the rate of volume decrease. During volume decrease, the blebs coalesced, paralleling the reduction in total cell volume and forming a rim around the cell.

Bleb formation appears to be a protective mechanism whereby excess volume is segregated until volume regulatory mechanisms can be brought into play.

Abstract Approved by: **Committee Chairman** _____

Program Director _____

Date 6/3/83 **Dean of Graduate School** _____

ACKNOWLEDGEMENTS

I would like to express special thanks to Dr. James A. Schafer, my advisor and friend, for his unlimited support and encouragement.

I would also like to express my appreciation to Dr. Don DiBona for his guidance and infinite patience. I thank Barbara Richey, Eugene Ames, and Sandy Silvers for sharing with me their expertise and for helping me to learn so many new techniques.

To Jim, my patient and loving husband

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF TABLES

INTRODUCTION

Life originated in an aqueous environment. As evolution progressed, living organisms were able to move out of the water, but the cellular environment remained mostly aqueous. Since water constitutes seventy to ninety percent of the volume of animal cells, regulation of cell water and therefore cell volume is a fundamental cell function which is essential for maintaining cell structure and the vascular supply.

A primary factor in cell volume control in higher organisms is the high water permeability of the plasma membrane. Since the osmolarity of the intra- and extracellular fluids are normally equal, intracellular volume has to be a function of the quantity of the solutes within the cell. Intracellular solute content is regulated across the selectively permeable plasma membrane by cellular metabolic processes and by movement along active, passive, and exchange pathways.

Many organisms, invertebrate as well as vertebrate, do not have the capability of osmoregulating their extracellular fluids, thus their extracellular fluids reflect environmental osmolarity. These organisms are referred to as osmoconformers. Many animals (particularly the euryhaline osmoconformers) encounter or live in an environment which imposes severe water stress such as high or fluctuating salinity, desiccation, or freezing, and their extracellular osmotic pressures may fluctuate widely (Yancey et al. 1982). When exposed to varying environments, they must rely on cellular volume and ion regulatory

processes to ensure survival. Maintenance of a constant intracellular volume in spite of changes in body fluid osmotic concentration is termed isosomotic intracellular regulation. Maintenance of an extracellular fluid osmotic pressure different from environmental osmolarity is referred to as anisosmotic regulation or osmoregulation. Isosomotic intracellular regulation has been observed in all marine invertebrates while anisosomotic regulation is an evolutionary advance seen in fewer organisms (Schoffeniels 1967).

Terrestrial animals have very effective extracellular fluid osmolarity control and must rely on cellular regulation only when extracellular osmotic control processes are overwhelmed or respond too slowly. Under normal conditions, metabolizing mammalian cells do not shrink or swell, even though most cells are freely permeable to water. However, some mammalian cells are continuously exposed to differing osmolarities. Saliva, sweat, and urine differ in osmolarity from the extracellular fluid from which they are derived. The cells which produce them of necessity lie between fluids of differing osmolarities and cannot be in equilibrium with both. Volume control mechanisms are necessary to protect these cells. Other cells may be intermittantly challenged by hypoxia, acid-base disturbances, electrolyte disturbances, temperature changes, or anisotonic stress and are dependent upon volume control mechanisms in these situations.

Because the intracellular protein concentration is higher than the extracellular protein concentration, mammalian cells must control total cation content to regulate volume (Tosteson 1964). According to current theory, steady state cell volume in most animal cells is regulated by balancing the rate of passive Na^+ diffusion into the cell (the leak) and active Na^+ extrusion from the cell (the pump) (Leaf 1970). The cell exerts this control through an active adenosine triphosphatase (ATPase) energized Na^+/K^+ transport pump (Skou 1957,

Post et al. 1960). Thus, the control and maintenance of the cell volume also requires a continuous energy supply. Cells swell and lose osmotically active solute if metabolism is impaired.

Cells also swell when subjected to hypotonic stress. In hypotonic media red blood cells expand from biconcave discs to spheres; with increasing stress the cells continue to expand until the cell membrane is disrupted (Hoffman 1977). Other cells have been assumed to react to hypotonic stress in a similar manner. However, increasing evidence indicates the behavior of red blood cells in hypotonic media is exceptional. Most mammalian cells react to hyptonic stress with a different type of swelling: the cell surface forms large blister-like protrusions called blebs. These blebs constitute incremental increases in cell volume which the cell must correct. Similar swelling through bleb formation is seen with other pathologic conditions and during some normal physiologic processes.

Volume adjustment during hypotonic stress is rather poorly understood, but more recent evidence, which is discussed below, indicates that it may not be entirely dependant upon the pump and leak mechanism.

The following sections will discuss morphologic changes and ionic movements associated with volume adjustment in hypotonic media. Special attention will be paid to these processes in Ehrlich ascites tumor cells.

CELL SURFACE CHANGES DURING STRESS

Blebs are blister-like protrusions seen on membrane surfaces during normal physiological processes and following cell injury or stress. Through the years these membrane formations have been given various names including balloons (Burrows and Neymann 1917, Hogue 1919), blebs (Lewis 1923), buds (Jackson 1927), blisters or potocytes (Zollinger 1948), bubbles (Costero and Pomerat 1951), pseudopods (Selby et al. 1956) and spicules (Weed and Bessis

1973). Most investigators have dismissed blebs as a curiosity or artifact. They apparently were first closely studied in 1923, when Lewis observed their formation along the edges of tissue cultured cells exposed to alkali. The blebs were formed on the cell surfaces, were filled with clear, homogeneous material, and rarely if ever contained organelles. In contrast, Jackson (1927) related bleb formation in kidney tissue to the acidic urine of rat kidneys rendered slightly "nephropathic" by chronic feeding of very high protein diets, but noted that "cellular injury" (unspecified) produced a similar change. Thus blebs had been found in both acidic and alkaline media. Jackson also described blebs in "uterine glands" during pregnancy. When Shear (1935) and Belkin and Shear (1938) reported their studies of cellular swelling in response to anoxia, they described blebs which appeared "underneath" the cell membrane within the first few minutes of swelling. However, they also found that bleb formation could be suppressed by adding colloids to the bath media.

In 1948, Zollinger used phase microscopy to study tissue slices in suspensions and found blebs on their free edges. He postulated that the blebs were an active function of living cells because they stopped forming when the cells died. Since these blebs were never seen in very fresh suspensions, they were most likely formed in response to anoxia. In his studies Zollinger observed that vacuoles were not related to bleb formation because vacuoles did not cause cell membrane bulges, appeared only after several hours in suspension, and were clearly distinct from blebs. He also observed that bleb formation was pH dependent. He found that agitation dislodged blebs from the cells, that temperature played no role in their formation, and that placing the tissues in distilled water accelerated bleb formation and growth. He called the process of bleb formation "potocytosis". Costero and Pomerat (1951) termed the process "zeiosis". They defined potocytosis as a process whereby blisters formed in

seconds to minutes, retracted over the period of ten to twenty minutes, and were separated from the cytoplasm by an interface membrane. Zeiosis was considered to be a much faster process and no interface membrane separated the blister and the cytoplasm.

Blebs have been described in many different types of cells and organisms. In addition to the agents already mentioned, blebs have been found to form in response to cooling (metabolic inhibition), viruses, ATP depletion, physical injury, inhibitors like cyanide (Chiang et al. 1968, Dellasega and Grantham 1973), cytochalasins, aldehydes (Davies and Stossel 1977) and heavy metals (Belkin and Hardy 1961, Chiang et al. 1968, Davies and Stossel 1977). Kitching (1934) observed blebs on the protozoan Cothurnia curvula bathed in hypotonic fluid. Costero and Pomerat (1951) observed blebs along the dendrites of growing nerve cells; Chiang and his colleagues (1968) on endothelial cells; Selby and his colleagues (1956) in tissues cooled to 0° C or in tissues mixed with viruses; Weed and Bessis (1973) on mammalian red blood cells depleted of ATP and exposed to alkali; Dornfeld and Owczarzak (1958) on cultured fibroblasts in the presence of ethylenediaminetetraacetic acid (EDTA) or with changes in localized ATP concentrations; and Schmid-Schönbein and his colleagues (1980) on white blood cells bathed in hypotonic media.

Lassen and his colleagues (1971) confirmed Jackson's observation (1927) that direct injury also caused bleb formation, and observed that during intracellular electrical recording blebs formed on Ehrlich cells at the site of microelectrode penetration. Donohoe and his coworkers (1978) used electron microscopy to study bleb formation in hypoxic renal tissue and found that the blebs contained many ribosomes but rarely contained larger organelles. Jewell and his colleagues (1982) noted that toxic chemicals caused bleb formation on isolated hepatocytes and postulated the blebs were related to changes in

extramitochondrial Ca^{++} concentration. Bleb formation has also been observed during mitosis. Boss (1955) found high concentrations of RNA in the area of bleb formation during the anaphase cycle of cell division while Lettré and his colleagues (1951) and Price (1967) related bleb formation to localized areas of high concentrations of ATP during the anaphase cycle.

Bleb formation in response to hypotonic shock has been observed on pituitary cells, myocardial cells, gastric cells, Ehrlich ascites tumor cells, and vaginal epithelial cells (Wilkerson, unpublished observations). The speed with which the blebs formed varied widely for the different cell types. Cardiac cells appeared to resist hypotonic media (98 mosm/kg) the longest, generally requiring thirty minutes or more before forming blebs. Ehrlich ascites tumor cell blebs were formed in less than a second. Persson and Spring (1982) found that volume changes associated with swelling in Necturus gallbladder epithelial cells bathed in hypotonic media were completed in less than ten seconds, but they did not describe blebs in these cells. All investigators who have described blebs have found that cells which have been swollen in hypotonic media resorbed their blebs and fully returned to their usual configuration when resuspended in isotonic solutions.

For many years, blebs were thought to form only on free cell surfaces, but Kessel (1968) and Wischnitzer (1970) noted the formation of multiple blebs on stalks on the cytoplasmic side of the outer membrane of the nuclear envelope. These blebs pinched off to form annulate lamellae. A mitochondrial bleb is evident in an electron micrograph of a chemically injured flounder tubule cell in studies by Trump and Ginn (1968) and by Laiho and Trump (1975). Blebs formed intracellularly or on cell surfaces in contact with other cells probably deform or flatten adjacent structures by compressing them, which might be the reason cell contact appears to reduce bleb formation (Rubin and Everhart 1973).

Although bleb formation and reduction appear to occur with specific cellular volume and solute changes, these events have never been quantitatively correlated. The primary object of this study was to correlate the morphologic changes and ion movements associated with volume adjustment in hypotonically stessed cells in order to establish the role of blebs in cellular volume control.

CELL VOLUME REGULATION

Traditionally, cell membranes have been considered freely permeable to water, and the movement of water between cells and interstitial fluids has been regarded as a consequence of osmolality differences. The capability for osmotic work has been accepted as a universal property of living matter, and cell volume has been considered to be regulated by mechanisms which control the distribution of solutes across the cell membrane.

During the early 1900's, cells were thought to contain nondiffusing macromolecules and ions which were responsible for water movement; cell swelling and lysis were thought to result from loss of impermeant characteristics. With the 1940's came a new approach to volume control. Both Danowski (1941) and Harris (1941) demonstrated that the cell membrane was permeable to cations and that their distribution was not static but was maintained by cellular metabolism. At that time, Dean (1941) first postulated active transport of Na^+ by cells. Since cells were obviously permeable to both Na^+ and K^+, they could not maintain a stable volume through cation impermeability and another mechanism was sought to explain why they did not swell and lyse. It was observed that inhibition of metabolism in various tissues produced swelling even though the tissues were bathed in isotonic media. Opie (1949) and Robinson (1950, 1953) described studies in which swelling induced by metabolic inhibition was prevented by placing tissues or cells in hypertonic salt solutions. These observations led to the theory that intracellular fluids had

higher osmotic pressures than extracellular fluids, and that the normal steady state was maintained by the continuous active extrusion of water from cells (Robinson 1950, Wilson 1954).

This interpretation could have been justified if osmotically active solutes did not move and swelling was due to shifts of water alone, but in 1951 Mudge demonstrated that the fluid entering the cells during swelling was not water alone but water and NaCl which was approximately isotonic with the medium. He found that inhibition of metabolism was associated with intracellular loss of K^+ and gain of Na^+, Cl^-, and water. Therefore, the swelling of tissues in chilled, buffered medium was more likely the result of inhibition of active transport of cations. Water movement appeared to result from the formation of osmotic gradients or the action of contractile vacuoles, pinocytosis, and electro-osmosis, and no conclusive evidence favored its active transport (Rorive and Gilles 1979). It is now generally accepted that a hypertonic medium prevents swelling in metabolically inhibited tissues because the increased concentration of neutral salts in the medium opposes the osmotic pressure exerted by the intracellular proteins (Leaf 1959).

By the mid-1950's isotope studies had confirmed the permeability of cell membranes to all osmotically active ions and solutes in the extracellular fluids. Wilson (1954) and Leaf (1956) suggested that metabolic energy was necessary for the maintenance of the Na^+ gradient and that active Na^+ extrusion was responsible for preserving normal cell volume because it counterbalanced nondiffusible cellular colloids. Since vertebrate cell cytoplasm and extracellular fluid were isosmotic, cell water content had to be determined by solute content, and therefore cell size had to be a function of solute content. This theory appeared to account for the reduction in the total number of osmotically active intracellular particles, and proposed a cell regulatory mechanism which was

sensitive to cell size and could assist in safeguarding the internal environment of the cell.

By the early 1960's the steady state maintenance of cell volume was considered to be satisfactorily explained by the action of a Na^+/K^+ exchange pump and leak working in parallel (Tosteson and Hoffman 1958, 1960). Accordingly, factors acting on either passive permeability or the cation pump would also affect the cell volume. The swelling seen with metabolic inhibition could be explained by pump interference which would allow Na^+ to follow its electrochemical gradient and Cl^- to accompany it, while K^+ leaked out. Since the Na^+ entry was greater than the K^+ loss, pump inhibition resulted in an isotonic gain of water.

While the pump and leak concept answered some questions about volume regulation it did not explain the role of intracellular compartmentalization of ions, specific membrane polarity, ion binding, water availability, and hydrostatic pressure brought about by contractile mechanisms (Kleinzeller 1965). It also did not explain how cells which lack Na^+/K^+ ATPase regulate their volume (Chan et al. 1964, Bonting and Caravaggio 1966, Klein and Breland 1966, Parker 1973, Gupta et al. 1974). However, the basic relationship implied by the pump leak concept was necessary on thermodynamic grounds to explain steady state volume and cation concentration in most cells (Kregenow 1981).

VOLUME REGULATORY DECREASE

The ability to reduce cell volume by extruding intracellular osmotically active solutes in dilutional states appears to be a general property of mammalian cells and is probably necessary for survival. If individual cells swelled in response to hypotonic stress, but had no means of controlling the swelling or reducing their volume, continued stress would lead to interference with cell

functions, deformation and compression of neighboring cells, and impairment of vascular supplies.

The cellular response to hypotonic stress is manifested by structural changes (bleb formation) which reflect rapid permeability and volume changes. These changes have a biphasic pattern: an increase in cell volume is followed by a decrease in cell volume. Cells exposed to hypotonic media initially swell as water enters the relatively hypertonic cell cytoplasm. However, these cells subsequently are able to reduce their volume, even in hypotonic media, by extruding solute. This phenomenon has been termed volume regulatory decrease. The speed and extent of volume regulatory decreases are different for different types of cells and also vary with the osmolality of the external medium. In all cells studied thus far, solute and water are both lost during volume regulation. However, volume regulation does not necessarily lead to full restoration of the original volume.

Since all cells appear to be able to regulate their volume, the fact that some cells, such as epithelial cells, respond more quickly than others may be a reflection of their greater exposure to environmental changes and the "vanguard role" they play in the battle for survival of the organism (Ussing 1978). Even plant cells undergo volume regulation and are able to regulate their internal ionic composition and turgor pressure in response to changes in external salinity (Bisson and Gutknecht 1975).

Swelling of tissues after hypotonic stress was described in 1733, when The Reverend Stephen Hales observed that intravenous injection of large amounts of water caused animal tissues to swell. However, volume regulation was apparently first described in 1932 by Lucké and McCutcheon, who observed that tissues swollen in hypotonic media tended to shrink after swelling. However, they attributed the shrinkage to cell deterioration. Not until 1965 did

Buckhold and his colleagues suggest that the shrinkage in mouse lymphoblasts which followed hypotonic swelling was a physiologic processs.

Since cell membranes are permeable, changes in external osmotic pressure should result in volume changes. Through the years cells have been known to behave somewhat like osmometers (Ponder and Saslow 1931, Darrow and Yannet 1935) but have not increased their volume to the extent predicted by osmotic theory. These deviations from the van't Hoff law have led to several postulates about the role of ions in cellular volume regulation (Dick 1971). These postulates included dependence of the osmotic coefficients of the cell solutes on concentration, elastic properties of the plasma membranes, binding of water, loss of osmotically active solute (Ponder and Saslow 1931, Kregenow 1971, Grantham 1977), or dilutional changes in net charges of nondiffusable macromolecules (Gary-Bobo and Solomon 1968). A rigid cell wall would be disadvantageous in mammalian cells because many of their vital processes are dependent upon a high surface-volume relationship. In most cases, tension at cell surfaces is very small. Therefore, the theory that volume regulation in mammalian cells is the result of hydrostatic pressure due to the elastic properties of the membrane is unlikely (Rand 1964). Evidence that loss of osmotically active solute during volume regulation is a cause for this discrepancy is provided by the observation that cells which have undergone volume regulation in hypotonic media shrink below their normal size when resuspended in isotonic media (Kitching 1934, Lang and Gainer 1969). Additionally, some investigators have neglected to allow for the osmotically inactive fraction of volume in their calculations. This error has been shown by Lucké and McCutcheon (1932) to result in the "appearance" of a less than perfect osmotic behavior.

In 1934 Kitching described both bleb formation and the subsequent volume regulatory decrease in hypotonically stressed protozoans Cothurnia

curvula. In addition, he observed that most ciliary movement stopped when the organism swelled in tap water but was resumed in some of the organisms during volume regulation, implying functional disturbances of contractile proteins during cell enlargement. (Kitching also noticed that the contractile vacuole ceased excreting when blebs were formed, but that contractions were increased when blebs were not formed, implying that bleb formation may also be related to contractile proteins.) More recently, Prusch (1977) has observed that the rate of contractile vacuole excretion varies inversely with external osmolality and is blocked by metabolic inhibition. Individual vesicles contain isosmotic fluid initially, but when fused with the vacuole, the fluid becomes hyposmotic with low K^+ and high Na^+ content.

Kleinzeller (1965, 1972) and Rorive and Gilles (1979) have proposed an energy requiring contractile process for volume regulation in which the filamentous structures of cells squeeze isosmotic solutions out of cells. Their system requires the mechanical forces resulting from cell elasticity and structural characteristics to operate with the pump and leak system to regulate volume in steady states and during stress. Actin-like Ca^{++} dependent ATPase contractile proteins are found in many animal cells and may play a role in a mechanochemical type of volume control (Hoffman 1977). This contractile mechanism would have to generate and sustain a hydrostatic pressure gradient large enough to offset cellular colloid osmotic pressure. However, Leaf (1970) has maintained that mammalian cells need a large surface to volume ratio for rapid exchange of metabolites and that cell motility requires a low surface tension. He has claimed that low surface tensions are possible because swelling is minimized by ion transport, and that the only other means to prevent or minimize cellular swelling would be to have a rigid cell wall. Additionally, Rand

and Burton (1964) were unable to find any significant hydrostatic pressure gradients across erythrocyte membranes.

In many organisms, the reduction of intracellular fluid osmolarity associated with volume regulation results from the loss of intracellular low molecular weight metabolic products such as free amino acids and their derivatives. Osmoconformers such as the blue crab have osmotic concentrations of approximately 1000 mosm/l within their muscle fibers (Schmidt-Nielson 1975), yet volume regulation by these fibers is accompanied almost exclusively by loss of nonessential amino acids with minor changes in ions. Changes in inorganic constituents appear to be of less quantitative importance (Lange 1964, Schoffeniels 1967, Gilles 1978). The increased permeability to amino acids during volume control processes may be related to changes in membrane configuration or to changes in ion concentrations. Starfish and sea urchins also undergo volume regulation in hypotonic media, although the sea urchin requires eight days to achieve its new steady state (Lange 1964).

Morphologic changes and volume adjustment in response to dilution are similar in the cells of invertebrates and vertebrates even though the changes in cell solutes appear to differ. In many vertebrate cells, volume readjustment is largely the result of changes in inorganic ion content, mostly K^+ loss. Little evidence suggests that cell amino acids play a significant role in volume regulation following anisosmotic stress in these cells. However, a minor modification of intracellular amino acid content does occur during volume regulation in toad skeletal muscle (Gordon 1965) and in Ehrlich ascites tumor cells (Hendil and Hoffmann 1974, Hoffmann 1978). Additionally, erythrocytes and heart muscle of fresh water adapted sea flounders undergo volume regulatory decreases with similar organic, ionic, and water shifts even though

their serum Na^+ concentrations are quite different in the two environments (Lange and Fugelli 1965, Vislie and Fugelli 1975).

Most vertebrate cells achieve a new steady state after completion of volume regulation in hypotonic media. Intracellular K^+ content is decreased and Na^+ content is normal or slightly increased. During volume regulatory decreases in duck and salamander red cells (Kregenow 1971, 1974, Siebens and Kregenow 1978), K^+ and Cl^- were lost, but no significant changes in Na^+ content occurred. In mouse tumor cells intracellular Na^+ content was normal, but K^+ content was decreased, even though K^+ permeability was normal and Na^+/K^+ pump activity was decreased (Roti Roti and Rothstein 1973).

The cardiac glycoside ouabain blocks ion movement across the Na^+/K^+ pump (Skou 1965). It prevents replacement of K^+ which has leaked out of the cells or extrusion of Na^+ which has leaked in and decreases the intracellular K^+/Na^+ ratio (Cala 1977). Ouabain has no effect on passive leak of K^+ (Sweadner and Goldin 1980) nor does it have an additive effect on cellular swelling when metabolism is inhibited before it is applied.

The effects of ouabain on the volume regulatory processes are uncertain. When ouabain was added at the time of hypotonic stress, cells were able to regulate their volume (Macknight et al. 1975, Siebens and Kregenow 1978, Persson and Spring 1982), and restoration of cell volume was as complete in the presence of ouabain as in its absence. Additionally, some studies have shown that when metabolism is restored to metabolically inhibited tissue in the presence of ouabain, the tissue is able to regulate its volume by extruding Na^+, Cl^-, and water. However, even though ouabain had no effect upon net water movement during volume regulatory decrease, by blocking the membrane pump it causes alterations in the intracellular K^+/Na^+ ratio. These findings have been interpreted as indicating that K^+ is not the main driving force in volume

adjustment, since the cell was able to volume regulate without reaccumulating intracellular K^+ (MacKnight 1968). On the other hand, some investigators have observed that preincubation of cells and tissues with ouabain does affect cellular volume control. Roti Roti and Rothstein (1973) observed that the addition of ouabain to cell suspensions at the time of exposure to hypotonic stress did not disturb the volume regulatory decrease, but that exposure of mouse leukemic cells to ouabain or K^+ free medium for several hours prior to hypotonic stress resulted in a diminished ability to regulate volume. The reason for the apparent discrepancy appears to be the different exposure times to ouabain and the difference in the manner the cells were prepared prior to introducing ouabain and hypotonic stress. Cells and tissues incubated in ouabain for several hours prior to hypotonic stress may be unable to volume regulate due to a specific action of ouabain. However, in studies in which an effect of ouabain on cell volume regulation was demonstrated, the cells were not allowed to equilibrate with the isotonic media and to warm to 37° C prior to the addition of ouabain. Therefore, it appears, more likely, that the inability of these cells to volume regulate is the result of hypoxia incurred during the preparation period.

Since the volume regulatory decrease in all cells is associated with both water and solute movement, some investigations have centered on the effects of ion replacement in the hypotonic media. Davson (1970), and Trump and Ginn (1968) found that normal cells and tissues swell in isotonic media containing high concentrations of KCl (137.5 mM). Kidney tubule cells bathed in this Na^+ free (K^+ replaced) isotonic medium not only swelled rapidly, but cytoplasmic organelles and particles were observed to undergo rapid Brownian motion (Trump and Ginn 1968) similar to that occuring in hypotonic media. Cells swell even more in Na^+ free hypotonic medium.

Persson and Spring (1982) observed that volume regulation in hypotonically stressed gallbladder cells did not occur in Na^+ free (K^+ replaced) medium, while Bui and Wiley (1981) reported similar findings with human lymphocytes. Lang and Gainer (1969) described inhibition of volume regulation in crab muscle fibers when they were bathed in Na^+ free (Li^+ substituted) medium. Reuber and his colleagues (1963) showed that hypotonically stressed frog muscle could volume regulate in the absence of external Cl^- but could not if Na^+ was replaced with K^+. Schmidt and McManus (1977) confirmed that volume regulatory decrease in duck erythrocytes did not occur in a low Na^+ medium. Kregenow (1971) postulated that the absence of the volume regulatory decrease in duck erthyrocytes suspended in low Na^+ (K^+ replaced) medium resulted from eliminating the K^+ electrochemical gradient. Bui and Wiley (1981) observed that eliminating the gradient by depleting intracellular K^+ also reduced the volume regulatory decrease.

It addition to Na^+ free media, hormones and other agents have been shown to alter volume regulation. Norepinephrine affects volume regulation in some cells, and metabolic inhibition affects volume regulation in all cells. Hypotonically swollen duck erythrocytes respond to norepinephrine by further swelling (Schmidt and McManus 1977). Norepinephrine appears to express its effects on the volume regulatory process indirectly through the mediation of the second messenger cAMP. Volume regulatory decrease in all cells studied has been blocked by hypothermia (Whittembury and Grantham 1976) and reduced by KCN; however, cyanide also reduced the amount of initial swelling, which suggests metabolic inhibition results in solute loss. However, these changes could reflect a selective loss of permeability to all solutes and not necessarily a decrease in available energy for a specific mechanism (Dellasega and Grantham 1973).

Various investigators have postulated mechanisms for the volume regulatory decrease. Kregenow (1971, 1974) suggested that duck red blood cells regulate their volume in isotonic and hypotonic media by readjusting their K^+ content with the Na^+/K^+ pump, and through a separate volume regulating mechanism which also controls the intracellular K^+ content. He proposed that during volume regulatory decrease, cell volume decreased due to K^+ loss induced by an elevated K^+ permeability. Cala (1977, 1980a, 1980b) studied volume regulatory decreases in hypotonically stressed flounder red cells and in amphiuma red cells and found that during volume regulatory decrease unbuffered suspension media became alkaline. Because of this alkalinization, he proposed that the volume regulatory decrease was activated by changes in intracellular pH and postulated electrically silent pathways for ion fluxes with a possible obligatory coupling of Na^+ and K^+ with H^+ and a Cl^-/HCO_3^- exchange. He suggested that with cell swelling, intracellular K^+ is exchanged for extracellular H^+ and that this K^+/H^+ exchange results in the initial alkalinization of unbuffered media during the volume regulatory decrease. Increased intracellular levels of H^+ in turn activate a Na^+/H^+ exchange and a Cl^-/HCO_3^- exchange.

In summary, volume regulatory decrease is a latent process that is not functionally expressed unless needed. It is a nonsteady state response that tends to restore normal cell volume. The role of Na^+ in volume readjustment is unclear at this time. In general, volume regulatory decrease is usually found to be ouabain insensitive; is sometimes blocked by diuretics such as amiloride or furosemide (Kregenow 1981); and in the absence of buffer, may alkalinize the external medium (Cala 1977, 1980a, 1980b, Kregenow 1981). There is some evidence that it may also be associated with functional disturbances of contractile proteins (Porter and Tucker 1981). Volume regulatory decreases are associated with some epithelial transport characteristics and with changes in

osmotically active intracellular solutes, mostly intracellular ions for vertebrates and organic molecules for invertebrates.

EHRLICH ASCITES TUMOR CELLS

The Ehrlich tumor line generates large numbers of genetically uniform cells (Charlton and Wenner 1978) and parallel studies can be done under widely differing conditions with minimal cellular variations. The cells have been used in an extensive variety of studies and their characteristics are well known. This tumor has been propagated longer than any other ascites tumor and its origin can be traced to one of nine solid transplantable mouse mammary carcinomas which Ehrlich and Apolant initiated in 1905 (Agnish and Fedoroff 1968, Lettré et al. 1972). The tumor is of epithelial origin (Yasuzumi and Sugihera 1958), is readily transmissible to other mice, and has a maximal life of twenty-nine to thirty-seven days in vivo in the mouse (Klein 1950). Noncycling cells retain their ability to cycle if the environment improves (Lala 1972). These cells contain organelles typical for most cells, and they can be directly, homogenously, and simultaneously exposed to injurous agents or fixative solutions; therefore, the pathophysiological effects of cell volume changes can be analyzed more precisely than is possible with tissues.

Bleb formation has been observed in cells during mitosis. Ehrlich cells undergo mitosis about every twenty-four hours and the permeability of their plasma membranes increases significantly during the mitotic cycle (Hempling 1972). Compartments of osmotically active and inactive water and solutes varied during the cell cycle while the water content remained constant (Hempling et al. 1981). This temporary increase in membrane permeability to ions appears to be the starting point for cell division and for a wide range of biosynthetic processes initiated by nonspecific agents (Malenkov et al. 1974).

It is not clear whether bleb formation involves an increase in cell surface area. The reason for the uncertainty is that the free surfaces of most Ehrlich cells are covered with microvilli. These microvilli have been postulated to constitute a membrane reserve available under certain physiologic conditions. Microvilli are usually seen on round, dividing, or partially spread cells in culture, but are rarely seen on fully spread cells in culture (Matsudaira and Burgess 1979, Follet and Goldman 1970).

Intracellular ion concentations of Ehrlich cells vary slightly under normal steady state conditions. This variation is most likely related to duration of intraperitoneal growth and environment; however, pre-incubation of cell suspensions in a physiologic solution for ten to thirty minutes (depending upon the cell age), allows the cells to equilibrate and adjust their ion concentrations to a uniform level. Additionally, the intracellular amino acid concentration of these cells is higher than the ascitic fluid; however, washing the cells in amino acid free buffer removes a large portion of the free amino acids (Laris et al. 1978).

Compartmentalization and binding may affect osmotically active intracellular solutes thus altering osmolarity and volume control. Many investigators have reported compartmentalization of intracellular ions (Pietrzyk and Heinz 1974). Both Na^+ and Cl^- were reported to be appreciably sequestered in the nuclei of these cells. The Na^+ content of the cytoplasm was found to be less than half of the overall cell Na^+ and approximately fifty percent of the Cl^- was thought to be in the nucleus (Pietrzyk and Heinz 1972, E. Heinz et al. 1977, Geck et al. 1980). However, these results subsequently have been contradicted by electron probe data (Jones et al. 1979, Dörge et al. 1981). Additionally neither Simonsen and Nielson (1971) nor Smith and Adams (1977) could confirm evidence for nuclear sequestration of Cl^-.

Both contractile elements (actin filaments) (Speth et al. 1981) and pH have been implicated in bleb formation and volume regulation processes. Ehrlich cells contain two types of filaments: thin ones in parallel bundles in the microvilli and in the cortical cytoplasm, and thicker filaments in loose strands and networks near the nucleus (Kristensen et al. 1973). In hypotonically stressed cells these microfilaments, microtubules, and intracellular membrane systems persisted for a longer time at pH 6.5 (Pentilla and Trump 1975a, 1975b). Actin in the microfilament bundles is thought to be active only at certain times during the cell cycle and in specialized regions of the membrane. It is also thought that microfilaments may be involved in motile activities and volume regulation (Goldman et al. 1975, Weihing 1976). Mitchell (1971) found that these cells do have the capacity for amoeboid movement. E. Heinz and his colleagues (1977) presented evidence that H^+ is actively transported out of the Ehrlich cell and that intracellular pH is essentially dependent on extracellular pH. Intracellular regions of high acidity created during metabolic activity are abolished when metabolism is inhibited (E. Heinz et al. 1977). Extracellular acidosis (pH of 5.8 to 6.5) appears to protect Ehrlich cells against the injurous effects of anoxia, heat, and aging (Penttila and Trump 1974b). The cells survive longer *in vitro* at this pH than at 7.4 and progression of cellular changes through various stages of cell injury at the ultrastructural level is slower. The lowered pH has been thought to affect the physical properties of the cell membrane by interacting with proteins or lipids to directly alter permeability, or to influence enzymes with rate limiting control of metabolic reactions to indirectly alter permeability (Penttila and Trump 1974a, 1975a, 1975b).

Volume Regulation in Ehrlich Cells

Ehrlich ascites tumor cells exhibit a biphasic volume response to hypotonic media. An initial period of rapid swelling is followed by a period of

slower volume decrease. Ehrlich cells react uniformly to volume changes induced by hypotonic stress and are able to partially regulate their volume when suspended in hypotonic media. These changes seem to reflect rapid water entry followed by increased K^+ and amino acid permeability. Individual cell volumes vary widely, but relative increases in volume are the same for cells of all sizes (Hoffmann 1978). Volume regulatory decreases in these cells are accompanied by loss of nonessential amino acids, KCl, and water (Hoffmann 1976, 1978). Rosenberg and his colleagues (1972) found the volume regulatory decrease in Ehrlich cells to be accompanied by a significant decrease in intracellular K^+ with little or no change in intracellular Na^+. In these studies they also observed that the volume regulatory decrease was reversibly inhibited by ouabain, K^+ free media, and Na^+ free (choline or Li^+ substituted) media and suggested that the Na^+/K^+ pump was involved in the volume regulatory decrease. Shank and his coworkers (1973) supported these findings.

Geck and Heinz (1980) observed that ions in Ehrlich ascites tumor cells are transported not only by a ouabain inhibitable Na^+/K^+ ATPase pump and by ion exchange and diffusion, but also by an electrically silent ternary symport mechanism which is referred to as a $Na^+/K^+/2Cl^-$ cotransport process. The $Na^+/K^+/2Cl$ cotransport facilitates inward movement of the ions, is ouabain insensitive, and is blocked by furosemide and similar diurectics (Wiley and Cooper 1974, Haas et al. 1982). Even though this cotransport represents only a small fraction of the total cation flux, some investigators (Geck et al. 1980, Haas et al. 1982) postulate that it is involved in cellular volume regulation because it is capable of increasing the amount of osmotically active ions in the cell and can lead to swelling. In this manner it would counteract the volume regulatory effects of the Na^+/K^+ pump. Cotransport is postulated to be activated by cell shrinkage and inactivated by swelling.

Intracellular Na^+ and K^+ concentrations appear to have a reciprocal relationship and in normal media their sum remains constant, but under stress Na^+, K^+, and water can change independently. In contrast to the above findings of Rosenberg and his colleagues (1972) and of Hoffmann (1977, 1978), King and his colleagues (1959a) found an initial increase in Na^+ with cell swelling. However, their studies involved cell injury rather than hypotonic stress.

Ehrlich cells are more permeable to water than they are to Na^+, K^+, or Cl^- (Hoffmann, 1978). Glucose or hypotonic dilution results in an increase in K^+ and Cl^- permeability and a decrease in Na^+ permeability, a decrease in Na^+/K^+ activity, and a net loss of K^+, Cl^-, and water (Hoffmann 1978). Laris and Henius (1982) observed that the addition of glucose to suspensions of Ehrlich cells causes a shrinkage similar to that seen during the volume regulatory decrease after hypotonic stress, a decrease in intracellular K^+, Cl^-, and water, and an increase in Na^+. This glucose activated system was partially inhibited by furosemide (twenty percent for the KCl loss and forty percent for the Na^+ increase). These investigators also observed that the volume regulatory decrease in response to glucose was sometimes followed by cell swelling.

MORPHOMETRY

Cells generally have complex shapes which may be determined by pressures exerted on their membrane and by their cytoskeleton. If a relationship between cell shape and cell function exists (Welling et al. 1978), quantitative morphologic studies could aid in evaluating and understanding transport phenomena.

The main purpose of this study was to correlate cellular morphologic and functional changes during hypotonic stress. Therefore, a system whereby changes in living cells could be observed during experimental manipulations was essential. By viewing cells under controlled physiological and non-physiological

conditions, volume changes were correlated with measurements made by other methods. Differential interference contrast microscopy was the basis of the morphometric system employed. This type of microscopy was developed by Nomarski (Allen et al. 1969) and provides three-dimensional images of living, unstained cells or tissue with high contrast, shallow depth of field, and good resolution. Contrast generated by interference in the sheared light beams provides structural detail when they are recombined. If a water-immersion objective lens is used, suspensions of living cells can be observed during experimental manipulation. DiBona (1978) developed this system and used it successfully to study amphibian urinary bladder and other transporting epithelia (DiBona et al. 1981). Spring and Persson (1981) used a similar system to investigate epithelial functional morphology.

Differential interference contrast microscopy provides greater resolution of structural detail than any other light microscopic method. Its extremely shallow depth of field (less than 0.5 μm for a 100X objective) is another sigificant advantage. Cells may be optically sectioned by focusing at different levels from apex to base; structures in one plane can be visualized with little inteference from structures at other levels of the cell (Allen et al. 1969). With this optical system, surface ridges and microvilli, as well as organelles such as mitochondria and nuclei can be distinquished.

Coupling interference microscopy with closed circuit television and a computer system allows digitization of images and accurate estimates of cell membrane areas in living preparations. Digitized pictures are sharp, can be stored for future use, can be redrawn as many times as desired, and can be measured rapidly. Accuracy is limited by the inability to exactly trace the membrane infoldings and microvilli of the cell, and by the resolution of the light microscope.

STATEMENT OF THE PROBLEM

In the present studies of the morphologic changes occurring in Ehrlich ascites tumor cells with volume control and their correlation with functional changes during hypotonic stress, the following issues were considered:

1. What is the structure of the blisters or blebs formed during hypotonic stress? Are the blebs bounded by plasma membrane? Is the membrane single or double? Is the membrane derived from existing membrane such as the microvilli or is it newly formed in response to stress?

2. Is there a membrane between the cell cytoplasm and the bleb interior?

3. Is the increased volume totally in the blebs or is it also in the cytoplasm of the cell? Does bleb formation and resorption parallel volume and ion changes?

4. What effect does the volume increase have on intracellular ion concentrations?

5. How does replacement of ions in the bath media effect bleb formation and the volume regulatory decrease?

6. Do inhibitors used to alter the volume regulatory decrease in other cells effect this mechanism in Ehrlich ascites tumor cells?

SUMMARY OF RESULTS OF THIS STUDY

Suspension of Ehrlich ascites tumor cells in hypotonic medium results in the formation of membrane protrusions (blebs) which are bounded on the external surface by plasma membrane. The contents of these blebs remain separated from the cell cytoplasm but there is no visible membrane barrier.

In the early stages of exposure to hypotonic medium, Ehrlich cells exhibit a biphasic volume response with initial cell swelling followed by a period of volume decrease. The volume regulatory decrease in Ehrlich cells is insensitive to ouabain, but is inhibited by either ethacrynic acid or the removal of Na^+ from the bathing medium. Bleb formation and resorption occur simultaneously with volume changes. The adaptation appears to involve selective permeability changes to both amino acids and ions. Cell Na^+ content changes are biphasic and follow water movements while K^+ content changes are largely in the opposite direction.

METHODS

All studies were conducted with Ehrlich ascites tumor cells as model mammalian cells. These cells can be grown and studied in isolated cell suspensions. Basically, all experiments involved the following manipulations. After harvesting and washing, cells were suspended in appropriate media to produce a cytocrit of approximately ten percent; after an initial incubation, the cells were diluted with either isotonic buffer (control) or distilled water (hypotonic shock). In all studies the final cytocrit of the suspension was 2.8 percent. Cell volumes and ion contents were measured and morphology was observed over a one hour time period. Additionally, the cells were examined by electron microscopy and, after immunohistochemical labeling of the membranes, by light microscopy (figure 1 and see below).

PREPARATION OF CELLS

Ehrlich ascites tumor cells of the Ehrlich Lettré line (hyperdiploid) were obtained in 1977 from Dr. Charles Levinson (University of Texas Medical School, San Antonio, Texas) and were maintained in this laboratory continuously by weekly intraperitoneal injections of 0.4 ml of tumor laden ascitic fluid into twenty to thirty gram female Swiss albino mice (Southern Animal Farms, Prattville, Alabama). Six to ten days after innoculation the mice were killed by cervical dislocation and tumor cells were harvested by exposing the peritoneum and aspirating the ascitic fluid. If the fluid was hemorrhagic, the aspirate was discarded.

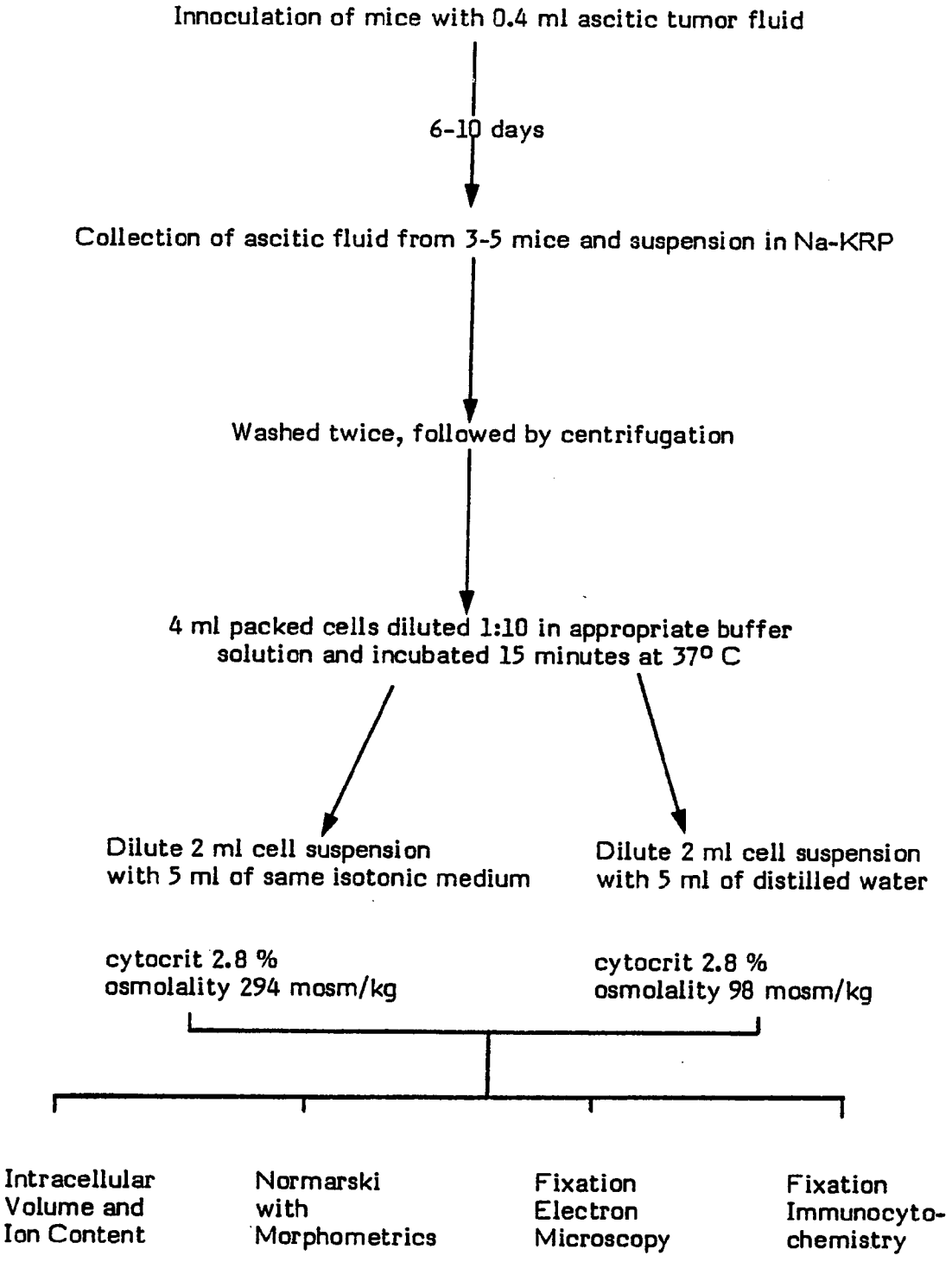


Figure 1. Experimental protocol.

A phosphate buffered Ringers solution (referred to as Na-KRP) was used for washing and resuspending the cells for most experiments. Its composition was 36.0 mM NaCl, 8.0 mM KCl, 1.5 mM $MgSO_4$, and 10 mM NaH_2PO_4/Na_2HPO_4. The Na-KRP solution was titrated to pH 7.40 with a small amount of 0.1 N NaOH, using an Orion Research model 701A/digital ionalyzer. All solutions had osmolalities of 290 to 300 mosm/kg when measured by the freezing point depression method (Advanced Osmometer Model 3W). Since $CaCl_2$ can contribute to cell clumping, and since it is not necessary for normal transport in these cells (Johnstone and Scholefield 1965, Levinson 1967, Smith and Vernon 1979), it was omitted in these studies. In pilot experiments comparing Na-KRP containing 2.0 mM $CaCl_2$ with Ca^{++} free Na-KRP, Ca^{++} did not alter the parameters measured. Morrill and his colleagues (1964) observed that Ca^{++} free media altered initial Na^+ and K^+ concentrations, but in the pilot Ca^{++} free experiments in the present studies the initial Na^+ and K^+ concentrations were not different from the values he obtained in media containing Ca^{++}.

The ascitic fluid was diluted in 20 ml of Na-KRP containing 100 U.S.P. units of sodium heparin. The resulting suspension was filtered through surgical gauze and centrifuged. Usually the ascitic fluid from three to five mice provided 4 ml of packed cells. The pellet was washed in Na-KRP and centrifuged two additional times in an International Model V centrifuge at 65 x g for ten minutes. The supernatant and top layer of cells were removed with suction and discarded after each centrifugation.

MEASUREMENT OF CELL VOLUME AND ION COMPOSITION

Ion concentrations and cell volumes were measured directly. After two washings the cells were resuspended in nine times their volume of either Na-KRP buffer or in buffer with the substitutions described in RESULTS. Two ml

portions of the ten percent cell suspension were pipetted into sixteen 25 ml Erlenmeyer incubation flasks equipped with detachable sidearms. The cell suspensions were incubated at 37° C in a water bath oscillating at 100 cycles per minute. The suspension depth was approximately 5 mm which together with the oscillation allowed good aeration. The sidearms were preweighed for later determination of pellet wet and dry weights, and each sidearm tube was filled with 5 ml of either buffer (nonstressed, control) or distilled water (stressed, hypotonic shock).

After the sidearms were filled, they were attached to the flasks containing the cell suspensions and all sixteen preparations were incubated at 37° C for fifteen minutes to allow the cells to equilibrate with the medium. All sixteen cell suspensions were mixed with their sidearm solutions simultaneously by tipping the flasks on a rack. The resulting suspensions had a volume of 7.0 ml and a cytocrit of 2.8 percent. The final osmolality of the control suspension was 294 mosm/kg and that of the hypotonic suspension was 98 mosm/kg. (The expected osmolality was 84, but ion movement was so rapid and the measurement of osmolality was relatively slow so that the osmolality had changed by the time a measurement could be obtained.) When Na-KRP buffer had been used to suspend the cells initially, the Na^+ concentration in the mixed suspension fell from 152 to 44 meq/l and the K^+ concentration decreased from 7.8 to 2.3 meq/l (figure 1). After mixing, the cell suspensions were returned to the flasks and the flasks were replaced in the shaking bath for additional incubation for varying times as described in RESULTS. Duplicate preparations for each time period were analyzed.

Extracellular Volume Determination

To determine cellular water content, extracellular space must be measured and this measurement usually involves the use of an impermeable

"marker". The basic assumption underlying the use of a marker to measure extracellular space is that the marker equilibrates completely in the extracellular fluid without penetrating the cell membrane. Inulin has proven satisfactory as a marker in many systems. Extracellular fluid volume in the pelleted cells was measured by adding dialyzed, tritium labeled methoxy inulin to each cell suspension one minute (twenty-five seconds in experiments of short duration) before centrifugation. The ^{3}H activity in the pellet extract provided an accurate measure of the trapped volume (McIver and Macknight 1974).

In the past, ^{14}C sorbitol has been used as an extracellular marker in studies with Ehrlich ascites tumor cells, but only after chilling suspensions to 4^{o} C (Schafer 1977, A. Heinz et al. 1981). Sorbitol slowly enters the intracellular space and would be unsuitable for use as an extracellular space marker during long incubation periods. In order to determine whether ^{3}H inulin could be used as an extracellular marker at the 37^{o} C temperature used in these studies, preliminary evaluations were done at 37^{o} C and at 4^{o} C, with various incubation times and cell suspension densities for both ^{14}C sorbitol and ^{3}H inulin. Tritiated inulin (generally labeled) with a specific activity of 100 to 500 mCi/g was obtained in crystalline form from New England Nuclear in batches of 5 mCi. A stock solution was made up by adding 5 ml of distilled water and dialyzing the solution against water for seventy-two hours. (The solution was redialyzed after two weeks.) For each study, a measured amount of the solution was evaporated under a stream of nitrogen and resuspended in the appropriate buffer. Generally labeled ^{14}C sorbitol was also obtained from New England Nuclear in batches of 0.25 mCi in 2.5 ml of aqueous/alcohol solution with a specific activity of 200 to 350 mCi/mmol. Two hundred and fifty μl of the ^{14}C sorbitol solution was evaporated and 5 ml of 290 mM unlabeled sorbitol solution was added to the residue.

Seventy μl of a solution containing 0.35 μCi of ^{14}C sorbitol and 1.0 μCi of dialyzed, ^{3}H-methoxy-inulin were added to each suspension of control or osmotically shocked cells. In some studies the markers were added one minute after rapid cooling of the suspension to between 0° C and 5° C. The suspensions were mixed thoroughly and rapidly by vortex and centrifuged at 2600 x g for five minutes in a Beckman Model J2-21 centrifuge. Other suspensions were allowed to incubate at 37° C for varying times in the presence of the extracellular markers to allow for their possible entrance into the cells. These suspensions of cells and marker were also vortexed and centrifuged for five minutes. In further studies, the cytocrit was varied and the markers were added at 37° C and allowed to incubate in an oscillating bath for ten minutes before centrifugation. Disintegrations per minute (dpm) were determined on aliquots of supernatants from the cell suspensions and from the aliquots of cell extracts after lyophilization.

The ^{3}H inulin or ^{14}C sorbitol activity was measured with a Packard Tri-Carb Model 3255 Liquid Scintillation Counter. An external standard channels ratio procedure was employed to confirm equal quenching in all samples. Supernatant and intracellular sorbitol and inulin concentrations were computed.

Multiple studies disclosed little uptake of either marker, and similar pellet extracellular volumes were obtained. Regression lines plotting the relationship between pellet wet weight and external cell water measured with sorbitol or inulin markers were significantly different. The slopes were not significantly different; therefore, the intercepts were not the same and the lines were parallel. The slope and intercept were 0.1540 $\pm$ 0.0054 (S.E.M.) and 0.0013 $\pm$ 0.0008 (S.E.M.) for inulin while the slope and intercept were 0.1849 $\pm$ 0.0095 (S.E.M.) and 0.0041 $\pm$ 0.0001 (S.E.M.) for sorbitol. The water volumes determined with inulin as a marker were always slightly lower than those measured with

sorbitol. These lower volumes probably indicate that some small extracellular or intracellular spaces were inaccessible to this polymer. Comparing the measured disintegrations of inulin and sorbitol indicated that inulin entered the intracellular space very slowly if it did so at all. In light of these considerations, the distribution of inulin was chosen as a measure of extracellular space. The extracellular space values obtained with inulin were less due to the lower intercept. The differences between the two markers had no practical significance within the bounds of these experiments.

Ion and Volume Measurements

At the end of the incubation period, the suspensions were tipped into the sidearms and the sidearms were quickly removed and centrifuged. The supernatants were poured off and saved for analysis of the extracellular label and ions. The tubes and their cell pellets were inverted over gauze for twenty minutes.

The internal walls of the sidearm tubes were dried to within 2 mm of the pellet using absorbent paper wrapped around a pair of forceps and the external surfaces were washed with petroleum ether. The tubes with their pellets were weighed to obtain the total wet weight, frozen at -40° C, and lyophilized overnight. The next morning the tubes were reweighed to obtain the total dry weight. The pellets were resuspended in 2 ml of LiCl (or distilled water if Cl^- measurements were to be made), and the suspensions were homogenized and allowed to stand ninety minutes at room temperature before being centrifuged again to sediment cell debris.

From the extracts, a 0.25 ml aliquot was diluted with 4.75 ml of LiCl for flame photometric analysis of Na^+ and K^+ Sodium and K^+ concentrations were determined in supernatants and in pellet extracts with an Instrumentation Laboratory Inc. Model 343 flame photometer (propane gas) calibrated with

standard solutions containing similar Na^+ and K^+ ratios. The Na^+ and K^+ were expressed in concentrations of meq/l solution of intracellular water or in μeq/g cell dry weight.

A second 0.25 ml aliquot was pipetted into scintillation vials containing 10 ml of scintillation fluid so that ^3H inulin activity could be measured. The scintillation fluid consisted of 4 liters of scintillation grade toluene, 15.6 g of 2,5-diphenyloxazole (PPO), 0.78 g of 1,4-di-2-(5-phenyloxazolyl) benzene (POPOP), and 273 ml Bio-Solv (BBS-3, Beckman Instruments, Inc.).

For Cl^- measurements, 0.5 ml of the pellet extract was pipetted into a solution containing 0.4 N HNO_3 and forty percent acetic acid for chloridometer analysis. The supernatant fluids from the centrifugation prior to lyophilization were diluted in the same manner, and intra- and extracellular Cl^- concentrations were determined with a Buchler Digital Chloridometer calibrated with standard solutions.

Cell volumes were determined by dividing the calculated weight of the intracellular water and measured weight of the dried cells by the number of cells. In three experiments the number of cells in a given suspension was determined with a Model ZBI Coulter counter (Coulter Electronic Inc.). Cells were harvested, washed and resuspended 1:10 in Na-KRP as previously described, preincubated in a 37° C water bath, and mixed with additional Na-KRP to a cytocrit of 2.8 percent. Twenty μl of this suspension was mixed with 10 ml of Isoton (filtered isotonic buffer, Coulter Electronics) and three drops of a lysing agent. Three counts were obtained for each experiment and the results were averaged.

Calculations

Pellet wet weight was determined by the difference between the total wet weight and the sidearm tare weight. Pellet dry weight was determined by

the difference between the total dry weight and tare weight. Total pellet water was determined gravimetrically as the difference between the total wet and dry weights. Extracellular space was calculated by two methods. The values obtained for intracellular water were divided by pellet dry weight. The resulting normalized value was called the swelling index.

Intracellular ion concentration calculations were based on the assumption that ions were uniformly distributed in the intracellular water. These concentrations were determined by the following relationship:

$$C_i = (B - C_{pe}W_e)/(W_t - W_e)$$

where:

B = total meq/l of substance in pellet

C_i = intracellular concentration of the substance

C_{pe} = concentration of the substance in the pellet extracellular volume (meq/l)

W_t = total pellet water (g)

W_e = pellet extracellular water (g)

Extracellular concentrations were measured as meq/l of solution, which was used interchangeably with meq/kg of water because the two units are not significantly different for solutions with 0.996 g of water for each ml of solution (Jacquez 1961).

When cells are incubated in a medium similar in ion composition to their own interstitial fluid, the derived intracellular K^+ is virtually unaffected by the size of the extracellular space because intracellular K^+ concentration is so much greater than that in the extracellular space. However, the derived intracellular water, Cl^-, and Na^+ contents are all markedly dependent upon the extracellular volume (McIver and Macknight 1974). Since pellets were prepared by dilution of one ml of incubated ascites suspension in 2.5 ml of Na-KRP

solution, the pellet extracellular concentration of the ion in question (C_{pe}) is two-sevenths of the original extracellular concentration. Therefore the correction for pellet extracellular contamination, $C_{pe}W_e$, becomes less critical. In addition, clustering of experimental points indicates that this method probably results in a good estimate of the intracellular concentration. For intracellular contents in μmol/g, $W_t - W_e$ was replaced in the calculations by pellet dry weight in grams.

MORPHOLOGY DURING SWELLING AND VOLUME REGULATION

Living and fixed preparations of control and experimental cells were closely examined so that artifacts introduced by the experimental studies could be identified and minimized. Living preparations were examined with differential interference contrast microscopy, and fixed specimens with light and electron microscopy.

Cells exposed to normal or hypotonic medium were examined by transmission and scanning electron microscopy. The cells were harvested, washed, and resuspended in Na-KRP. After incubating for fifteen minutes in a shaking water bath at 37° C, the cells were mixed with an additional 5 ml of either Na-KRP or distilled water as previously described. The suspensions were allowed to incubate an additional one or thirty minutes.

Transmission Electron Microscopy

Cells used for transmission electron microscopy were fixed by adding enough gluteraldehyde to make a one percent solution. The mixture was allowed to stand at room temperature for forty minutes. By this time the cells had settled to the bottom of the container and the supernatant fluid could be removed with a pipette. A one percent osmium tetroxide solution was mixed with the cells and the container was closed and placed in a hood. (These two fixatives were prepared in media with ion concentrations similar to extracellular

fluid to avoid concentration gradients and other possible effects of ions in the non isotonic conventional electron microscopy fixatives. The fixative buffer used was Na-KRP mixed with distilled water so that its osmolality was two-thirds the osmolality of prefixative buffers.) After one hour, the supernatant was removed and the cells were successively washed and suspended for periods of five minutes in fifty, seventy, and eighty percent ethanol. Next the cells were twice washed and suspended in ninety-five percent alcohol for ten minutes followed by an identical washing and suspension in one hundred percent alcohol. The cells were then suspended in propylene oxide for two fifteen-minute periods. Finally they were placed in Spurr epoxy for at least one hour and allowed to settle to the bottom of the vials.

The epoxy was removed by pipette and the cells gently lifted from the vials with a rounded stick, placed in fresh epoxy, and allowed to stand overnight in a 60° C oven to polymerize the resin. The specimens were removed from their superficial plastic casings and prepared for sectioning with a single edge razor blade. Both thick sections (one micron) and thin sections were cut with a Reichert Om-U2 heat advanced microtome. The thick sections were stained with toluidine blue for thirty to one hundred seconds, washed with distilled water, and covered with a cover slip. The thin sections were spread on 300 mesh copper grids that had been washed in $CHCl_3$ by passing cotton swabs wet with $CHCl_3$ over the knife basins before and after the sections had been attached to the grids. The sections were stained with uranyl acetate (one part super saturated uranyl acetate to one part alcohol) for twenty minutes, rinsed with fifty percent alcohol, rinsed with distilled water, and blotted dry (Swift and Rasch 1958). In addition, the sections were stained in lead citrate for twenty minutes and rinsed in NaOH, followed by several distilled water rinses and a final blotting. Thin

sections were examined with a Phillips 201 electron microscope. Electron micrographs were taken with Kodak electron microscopy film 4489.

Scanning Electron Microscopy

For scanning microscopy the cells were washed, incubated, and mixed with either Na-KRP or distilled water. The cells were fixed on Whatman 1.2 micron filters and dehydrated with a graded acetonitrile series. After a fifty to sixty second agitation to allow for mixing, the suspensions were placed on filters and immediately layered with one percent glutaraldehyde. The glutaraldehyde layer was maintained for twenty minutes, after which the cells were washed with buffer solution three times. The filter papers were placed in petri dishes and covered with osmium tetroxide for an additional twenty minutes, then dipped in buffer several times and placed in fifty percent acetonitrile for three to five minutes. Subsequently the filter paper and cells were placed in seventy percent acetonitrile for three to five minutes, in ninety-five percent acetonitrile for three to five minutes twice, and in absolute acetonitrile for fifteen minutes during which the absolute acetonitrile was changed twice. The filter and specimens were dried in a critical point drier (Balzers Union, model CPD010) and sputter-coated with gold:palladium (Technics Hummer). The scanning microscope was a JEOL 100CX TEMSSCAN and micrographs were taken with a Polaroid Land 545 camera.

Immunocytochemistry

To further characterize the membrane of the blebs, immunocytochemical techniques were used to identify specific membrane proteins. Immunocytochemistry provides a method for locating specific proteins in biological tissue by the reaction between antibody and antigen. Immunoperoxidase can be used to selectively label the antigen-antibody sites.

Both immunoglobulin G (IgG) and ATPase on the plasma membrane surface were identified with peroxidase-antiperoxidase (PAP) labeling techniques.

The first stage of the process entails applying two agents successively to the specimen for the purpose of blocking interference from naturally occurring cell constituents. Each of these blocking reagents is washed off. In the second stage, the antigen is located and labeled by the successive application of three separate antibodies. The first antibody is specific for the antigen being sought in the specimen. The second or link antibody is produced against gamma globulin or a more specific antibody protein of the animal which made the first antibody and binds to the first antibody. The third or labeling antibody comes from the same animal species as the first, is reactive with the link antibody, and is combined by an antigen-antibody reaction with the molecules of peroxidase necessary for the development of color at the antigen site. In the final step, the labeled site as well as the general cell morphology is made visible.

The cells were harvested, washed, and resuspended in Na-KRP. The control and experimental suspensions were mixed with buffer or distilled water and a drop of the resulting suspension was lightly smeared immediately on a slide which had been prepared previously for good adhesion (by dipping in 0.5 percent Elmer's glue-all (Borden, Inc.) and heat-fixing for thirty minutes at 60° C). The slides were immersed as rapidly as possible in B5 fixative (90 ml of hot distilled water, 6.0 g mercury bichloride, and 1.25 g anhydrous sodium acetate, to which 10 ml of 37.5 percent formaldehyde was added just prior to use) and allowed to fix for ten minutes. The slides were rinsed in ninety-five percent ethanol for one minute, followed by water for one minute. Next the slides were immersed in Lugol's iodine solution (1.0 g iodine, 2.0 g potassium iodide and 100.0 ml of distilled water) another minute to remove the mercuric chloride, washed in distilled water, immersed in two percent sodium thiosulfate for one minute to

remove the iodine, again washed in distilled water, and finally placed in a phosphate buffer (referred to as PBS) containing 8 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KCl, 0.2 g KH_2PO_4, and distilled water to one liter, pH 7.6 $\pm$ 0.2.

The slides were kept moist throughout the PAP procedure by placing them in a moist chamber. Three percent hydrogen peroxide, a blocking agent to mask endogenous peroxidase activity, was placed on the slides for five mintutes and followed by a PBS wash for three to five minutes. Next a ten percent solution of rabbit serum (in four percent bovine serum albumin/Tris buffer, pH 7.4) was applied to block non-specific binding of antibody protein molecules. After a twenty minute immersion, the serum was gently tapped from the slide.

Primary antibody to hog ATPase with previously demonstrated cross reactivity to rabbit and rat (supplied through the courtesy of Drs. Adam Smolka and George Sachs, Laboratory of Membrane Biology, University of Alabama in Birmingham) was diluted 1:10, 1:100, 1:500, 1:1,000, 1:5,000, and 1:10,000. The primary antibody was placed on the slide for twenty minutes and was followed by a wash with PBS for ten minutes. The link antibody (rabbit antimouse IgG, lot 2001, Sternberger-Meyer Immunocytochemicals, Inc.), diluted 1:40 with ten percent rabbit serum in PBS, was allowed to remain on the slide for twenty minutes. Following a wash in PBS for ten minutes, the cells were covered for twenty minutes with peroxidase-antiperoxidase conjugate (PAP, mouse origin, Sternberger-Meyer Immunocytochemicals, Inc.). The PAP was diluted 1:80 with ten percent rabbit serum in PBS. The slides were again washed for ten minutes with PBS, and amino-ethylcarbasole (AEC) substrate was applied for forty minutes and washed off briefly. A three minute immersion in Mayer's hematoxylin counterstain (Sigma Chemical Company) was followed by a wash until clear and a two minute immersion in ammonia water. A cover slip was placed on the slides using Aqua-Mount (Lerner Laboratories).

Hog stomach (also supplied through the courtesy of Dr. Sachs) was used as a positive control. Negative controls for cells incubated in either Na-KRP or distilled water were prepared by substituting antibody to chorionic gonadotropin for the primary antibody.

Morphometric Analysis

Cells treated with normal or hypotonic medium were examined by differential interference contrast (DIC or Nomarski) microscopy to visualize in vitro volume changes without fixation artifacts. Video images were recorded or photographs were taken for later morphometric analysis. The greater sensitivity of video detection methods permitted the recording of high quality images without intense illumination, so that specimen temperature was not increased to nonphysiologic levels. Changes in cell outline and cell volumes were measured with computer assisted morphometrics. The measurements of parameters were subjected to statistical evaluation.

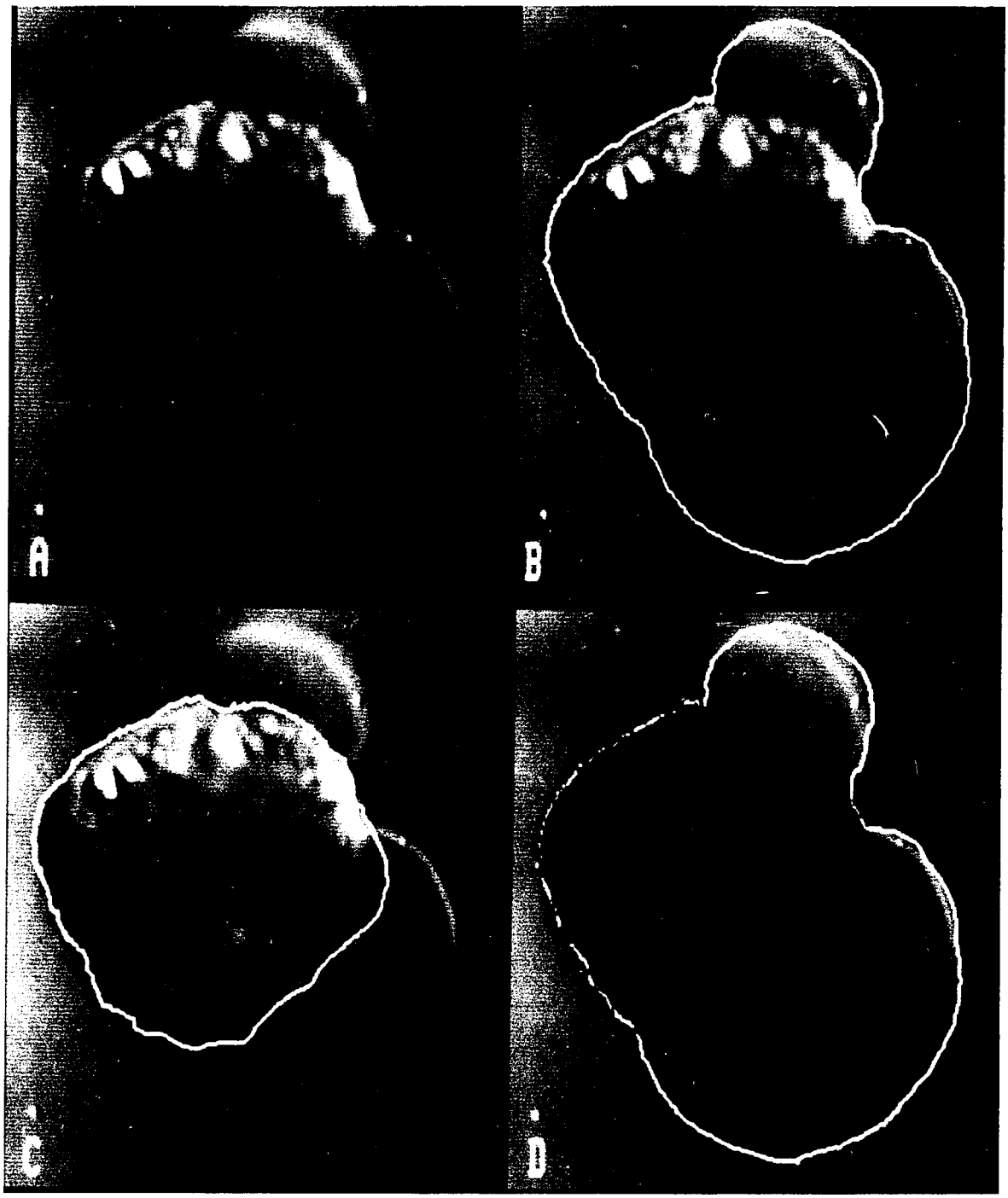
For Nomarski microscopy, a modified Zeiss IM-35 inverted microscope (Carl Zeiss) was used, with the standard DIC condenser assembly replaced by a high numerical aperture water-immersion objective lens with a Wollaston prism mounted in its back-focal plane (Zeiss Acromat 40/0.75). This lens has a narrow barrel, tapered to 9 mm at the front edge, and can easily be brought down into a suspension of cells. The system relies on the interference of a pair of wavefronts to generate contrast. When the sheared beams are recombined, structural details are revealed by the contrast generated by interference as the light beams pass through the specimen. The rate-of-change or gradient of refractive index change along the direction of beam shearing determines the intensity of the contrast generated by an object (DiBona 1978, Spring and Persson 1981).

Photomicrographs were taken at 320X magnification under oil immersion with transmitted light using a Contax 35 mm camera.

All morphometric studies were made on cells diluted in the manner described previously and were observed by Nomarski microscopy at approximately one minute and thirty minutes after mixing. The one minute time period was chosen because the cells have usually reached their peak volume in less than one minute. By thirty minutes the cell volumes appeared to be approaching a steady state. The constraints of the experimental system required the video images for the short-time experiments to be digitized and recorded between fifty and seventy-five seconds after dilution. These cells were diluted and immediately placed on a cover slip over an inverted 100/1.3 oil immersion objective lens and focused. In the other series the cells were incubated at 37° C for thirty minutes after dilution and then video images were recorded.

The video images of the cells were digitized and recorded with a Grinnell (GMR-27) computer-based (PDP11/23, Digital Equipment Corporation) digitizer. The area of the coverslip specimen derived from the primary suspension was considerably larger than the fields which could be examined microscopically at useful magnifications. Stochastic subsampling of the individual coverslip preparations was achieved by making random displacements of the mechanical stage of the microscope. By moving a cursor, cell outlines were traced and recorded on a video screen (figure 2). All of the cursor coordinates were stored in computer memory, allowing areas and perimeters to be calculated or cell outlines to be redrawn at any time. The focal plane chosen was the one that gave the maximum cell dimensions. No effort was made to include microvilli in the cell tracing. The hypotonically stressed cells were traced twice, (figure 2B and 2C) with the first tracing including the blebs, and the second just the apparent cell periphery without the blebs.

Figure 2. Composite of digitized cells for tracing cell outlines. 2A Cell. 2B Cell and blebs outlined. 2C Cell body only outlined. 2D Bleb outlined. Magnification, 1280X.


A
B
C
D

The area and perimeter bounded by the outlines were directly derived through numerical integration by the computer. These measurements were in units of pixels. Video images of geometric figures of known dimensions gave a ratio of 34.917 pixels per square micron for converting pixels to microns and square microns. Repeated measurements of the same cell had a coefficient of variation of less than 2.8 percent.

These measurements allowed comparisons to be made between the control cells and the hypotonic cells to ascertain whether the entire volume increase with hypotonic shock could be accounted for by the bleb volumes. They were also used to compare the morphometric measurements with the volumes measured by the tracer studies described above.

The cell surfaces were treated as if they were smooth when the membrane was actually highly convoluted and irregular. Volumes were calculated for the cells as if they were spheres and ellipsoids as described in RESULTS. The index of circularity was 0.939 which is compatible with an ellipsoid shape.

Biochemicals

Ethacrynic acid, ouabain, colcichine, N^6O^2-dibutyryladenosine 3':5' cyclic monophosphate (Bu_2cAMP) were obtained from Sigma Chemical Company. Furosemide was obtained from Hoechst-Roussel Pharmaceuticals, Inc. and amiloride was obtained from Merck, Sharp, and Dohme Company.

Statistical Analysis

The data are presented as means with the standard deviation appended. Whenever the standard error of the mean is appended, the notation (S.E.M.) is used. The significance of differences was determined by either a paired or unpaired Student's t-test. Significance was not attributed to the difference between any two means unless the t-test calculation had a P value < 0.05.

RESULTS

These experiments were designed to correlate morphologic changes occurring during experimental manipulation with some of the basic physiologic functions of Ehrlich ascites tumor cells. Control cells maintained a relatively constant volume and K^+ content during the studies; except for some initial scatter, the Na^+ content of these cells was also constant. In contrast, the hypotonically shocked cells swelled rapidly to almost three times their initial volume. This swelling subsequently declined gradually toward--but never reached--basal levels. Initially, the K^+ content of the stressed cells declined while the Na^+ content rose; however, during the early period of volume adjustment, these ions moved toward their basal levels. Morphologic changes occurred simultaneously. During volume increase, small protrusions or blebs appeared randomly on the cell surface. During volume adjustment these blebs coalesced and decreased in volume. The morphologic changes and ion and volume changes were closely correlated and are described in detail.

MORPHOLOGY DURING SWELLING AND VOLUME REGULATORY DECREASE

With ordinary bright field microscopy, fixed and stained tissues appear as two dimensional static images. The only details visualized are those revealed by staining differences. Electron microscopy is limited to fixed and stained tissues, but the two dimensional images include fine structural details which cannot be seen with any other method currently available. However, artifacts can be introduced by the specimen preparation procedures. Interference

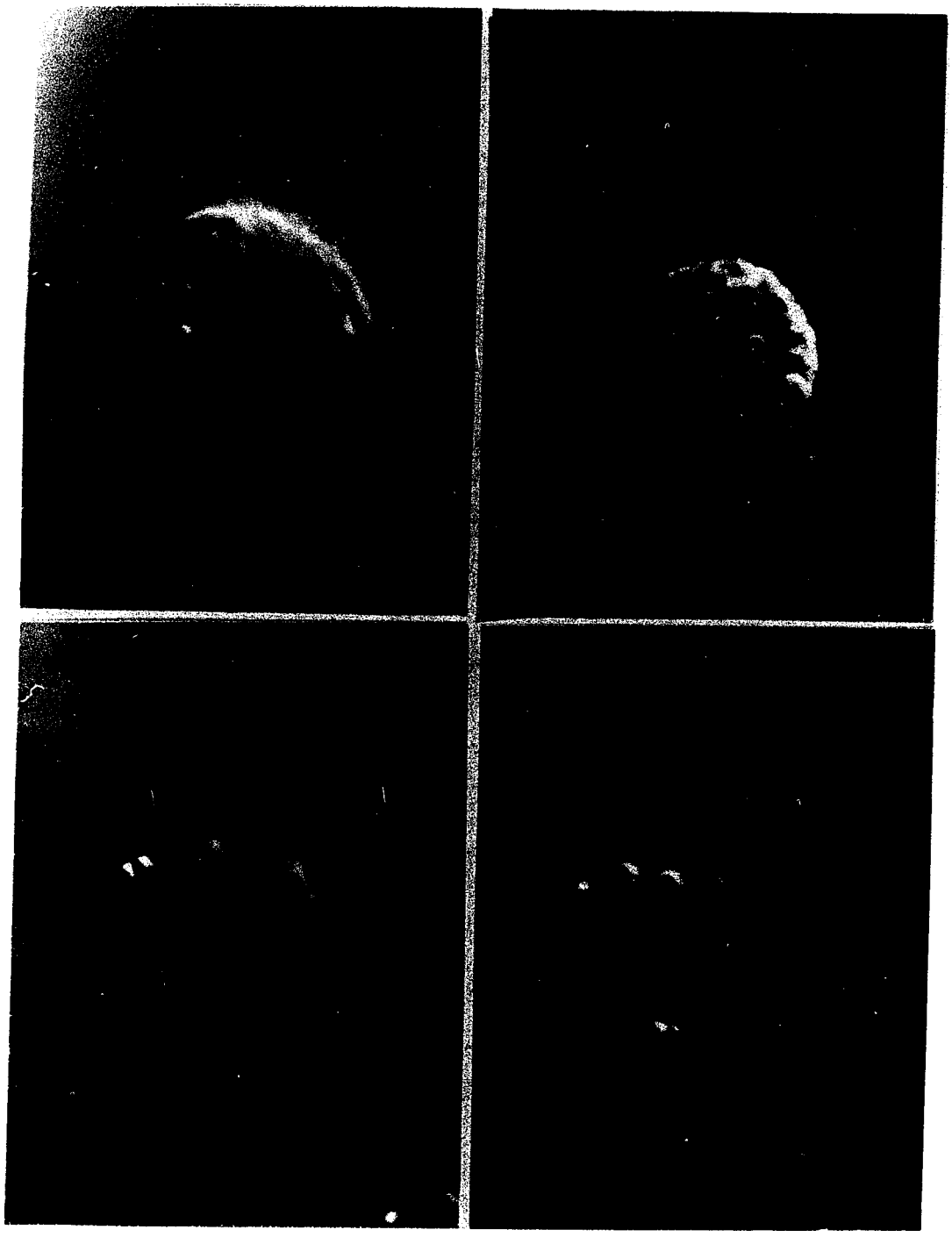
contrast microscopy allowed the examination of vital cells and eliminated the problem of fixation artifact.

Interference Contrast Microscopy

Control cells at one minute and at thirty minutes after dilution can be seen in figures 3A and 3B. At one minute the cell radii varied between 4.5 and 11.2 microns. Well-developed microvilli on the surface of the cell also varied in size. Numerous mitochondria and other unidentified organelles could be seen throughout the cytoplasm. Usually one to three highly refractile organelles, which were assumed to be lipid bodies, were also visible. One or more pleomorphic nuclei occupied approximately thirty percent or more of the cellular volume. A prominent nucleolus was usually present. Control cells were roughly spherical in shape, as has been observed by other investigators (Ambrose 1966, Maizels et al. 1958a, Hempling 1958). (The spherical form requires the least surface energy and is usually assumed by cells in suspension.) Thirty minutes after dilution with Na-KRP the cells, their microvilli, nuclei, and organelles appeared unchanged. However, as discussed below, the number of lipid bodies and the cell radius were both decreased.

Hypotonically stressed cells one and thirty minutes after dilution are illustrated in figures 3C and 3D. When cell suspensions were mixed with distilled water, membrane alterations were present on all cells by the time the cells could be placed on a coverslip and focused. In several experiments, cells were brought into focus prior to hypotonic stress; the visual changes were estimated to begin in less than a second. The cell membrane appeared to bulge at random sites in a manner best described as explosive ballooning. The protrusions (blebs) appeared to be externally surrounded by cell membrane and were spherical in shape with a constricted neck. The cytoplasm in these blebs was much less dense than the cytoplasm of the remainder of the cell and appeared clear. (Other investigators

Figure 3. Composite of cells at maximum diameter as seen with Nomarski optics. Four different cells are shown. 3A is a cell suspended in isotonic solution for one minute. 3B is a cell suspended in isotonic solution for 30 minutes. 3C is a cell hypotonically stressed for one minute and 3D is a cell hypotonically stressed for 30 minutes. (The focus in figure 3D was on the microvilli and therefore the cell outline is not in sharp focus.) Magnification, 1280X.



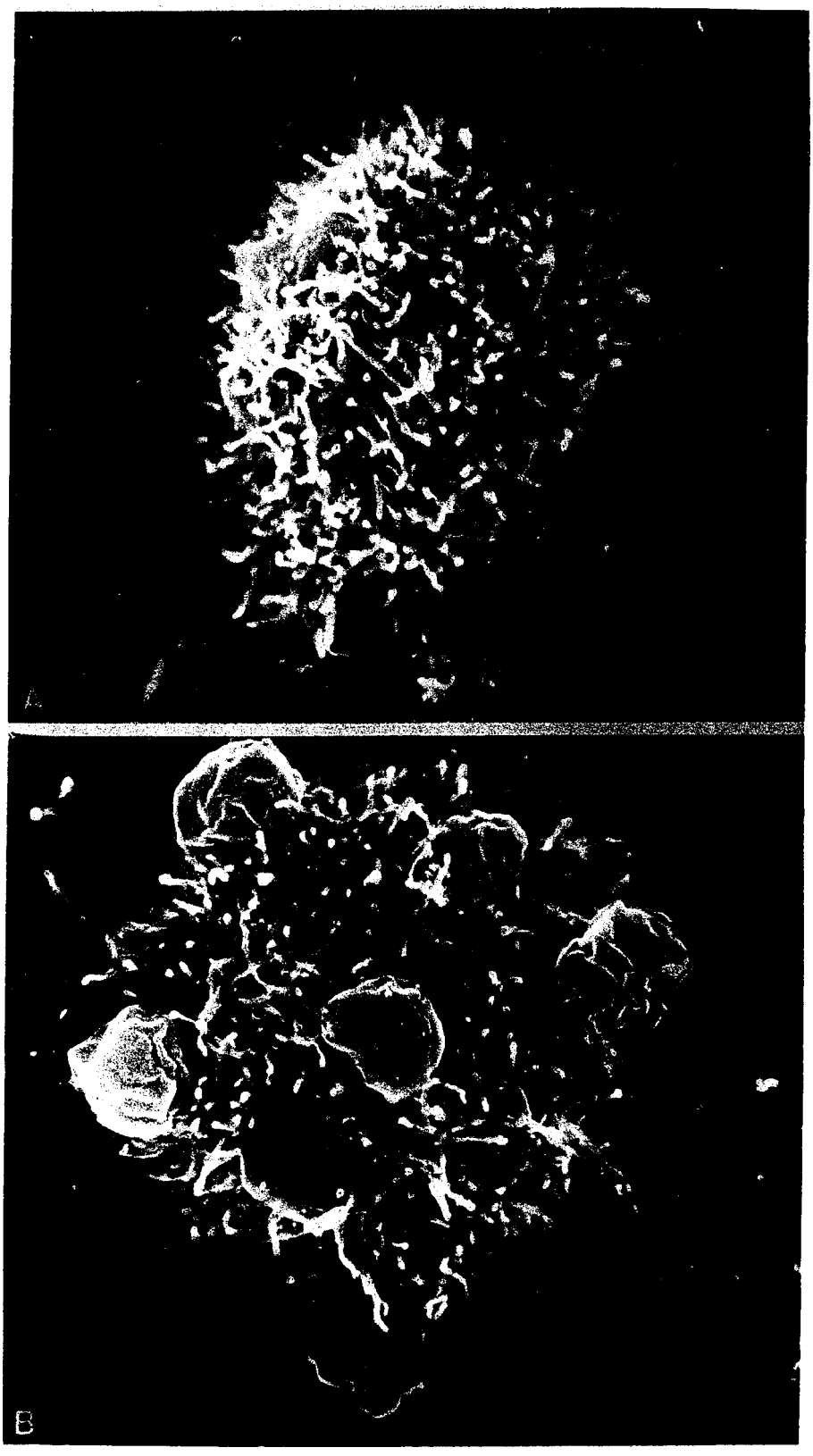
also have seen rapid bleb formation when cells were exposed to hypotonic media (Persson and Spring 1982).) The blebs were randomly distributed on the membrane surfaces and were not uniform in size. Their outer membranes appeared to be continuous with the plasma membrane. Nomarski microscopy did not provide sufficient resolution to determine whether the cytoplasm of the bleb was physically separated from the cytoplasm of the remainder of the cell by a membrane, but the bleb contents were distinctly separated from the rest of the cell. Rapid Brownian motion of organelles was commonly observed in many cells, and an occasional mitochrondrion appeared to "shoot" into the blebs from the cell cytoplasm. Numerous lipid bodies appeared to have been ejected from the cells, and were seen floating in the extracellular fluid.

With continued incubation in hypotonic medium, the blebs initially grew in size and could be seen to coalesce. At thirty minutes the blebs had decreased in volume and no longer protruded from the cell surface. Instead, the cell outline had become spherical, and the less dense cytoplasm associated with the blebs surrounded the denser cell cytoplasm, separating it from the cell membrane. Microvilli were numerous and prominent although not as numerous as prior to the hypotonic stress.

Scanning Electron Microscopy

The control cells as seen with scanning microscopy were generally spherical in shape and covered with numerous microvilli at both one minute and at thirty minutes (figure 4A). These microvilli were generally thin and their length varied, confirming the observations made with Nomarski optics. This appearance was dramatically altered within one minute after exposing the cells to hypotonic media: the cells appeared to be covered with both blebs and distended microvilli (figure 4B). At thirty minutes after hypotonic challenge the blebs appeared to cover most of the cell surface and small infoldings or recesses

Figure 4. Scanning electron micrographs of isotonic (control) cells, figure 4A and of hypotonically stressed cells, figure 4B. Cells were diluted with isotonic Na-KRP (4A) or with water (4B) one minute before fixation. Magnification, 5000X.


A
B

on their surface suggested they were becoming flaccid. The microvilli generally appeared less swollen. Some cells were completely covered with blebs that contained infoldings and recesses, and the cell appeared spherical.

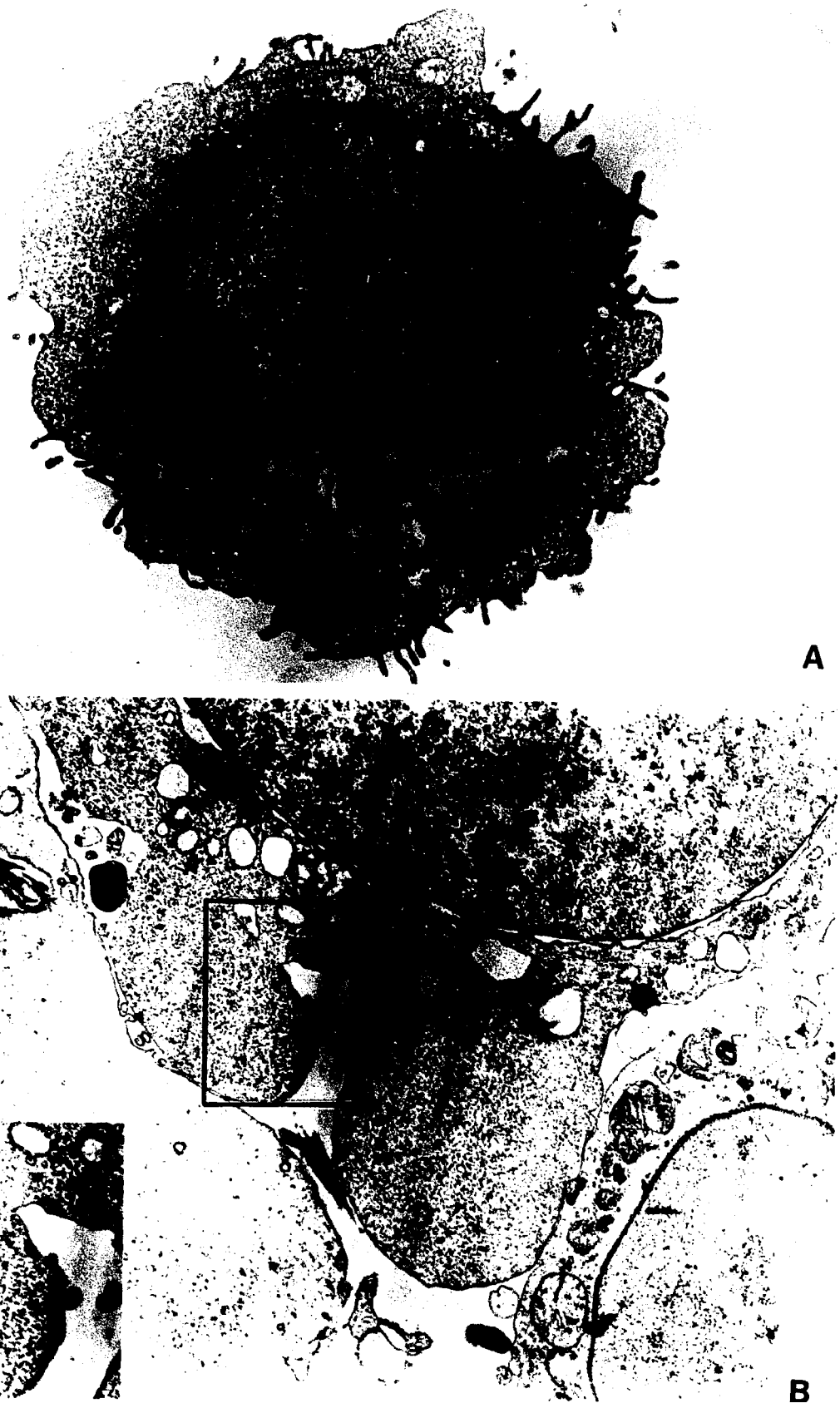
Transmission Electron Microscopy

When observed with Nomarski optics the bleb membrane appeared to be continuous with the plasma membrane on its external surface and there appeared to be a structural division such as a membrane separating the cytoplasm of the cell and the bleb (figure 3C). Cells were studied with transmission electron microscopy to determine whether the bleb membrane was indeed continuous with the plasma membrane and to closely examine the internal bleb boundary.

Control cells were observed to have prominent microvilli extending from the membrane surface and to have a roughly circular profile (figure 5A). The cytoplasm was relatively dense with numerous free ribosomes and both smooth and rough endoplasmic reticulum. Lysosomes, microtubules, scattered filamentous mitochondria, and several large lipid bodies were present. A golgi apparatus was usually located close to the nucleus. The cells contained one or more relatively large pleomorphic nuclei with prominent porous nuclear membranes. Cells were observed in various stages of cell division. Other investigators have found similar ultrastructural morphology (Herdson and Kaltenbach 1966, Beams and Kessel 1968).

After only one minute in hypotonic media, definite structural changes were present in the cell membrane (figure 5B). The number of microvilli appeared smaller, their size was reduced, and blebs were present. In the cytoplasm, the quantity of smooth and rough endoplasmic reticulum was diminished. Several large vacuoles were found, whereas vacuoles were uncommon in control cells and when present were much smaller. Herdson and Katenbach found extensive vacuolization but no blebs in their electron

Figure 5. Transmission electron micrographs of both isotonic (control), figure 5A and of hypotonically stressed cells, figure 5B. Insert contains higher magnification of the base of the bleb to demonstrate the absence of structural separations of the bleb and the remaining cell cytoplasm. Cells were diluted with isotonic Na-KRP (5A) or water (5B) one minute before fixation. Magnification, 5,000X (A), 10,600X (B), and 13,250X (Insert).


A
B

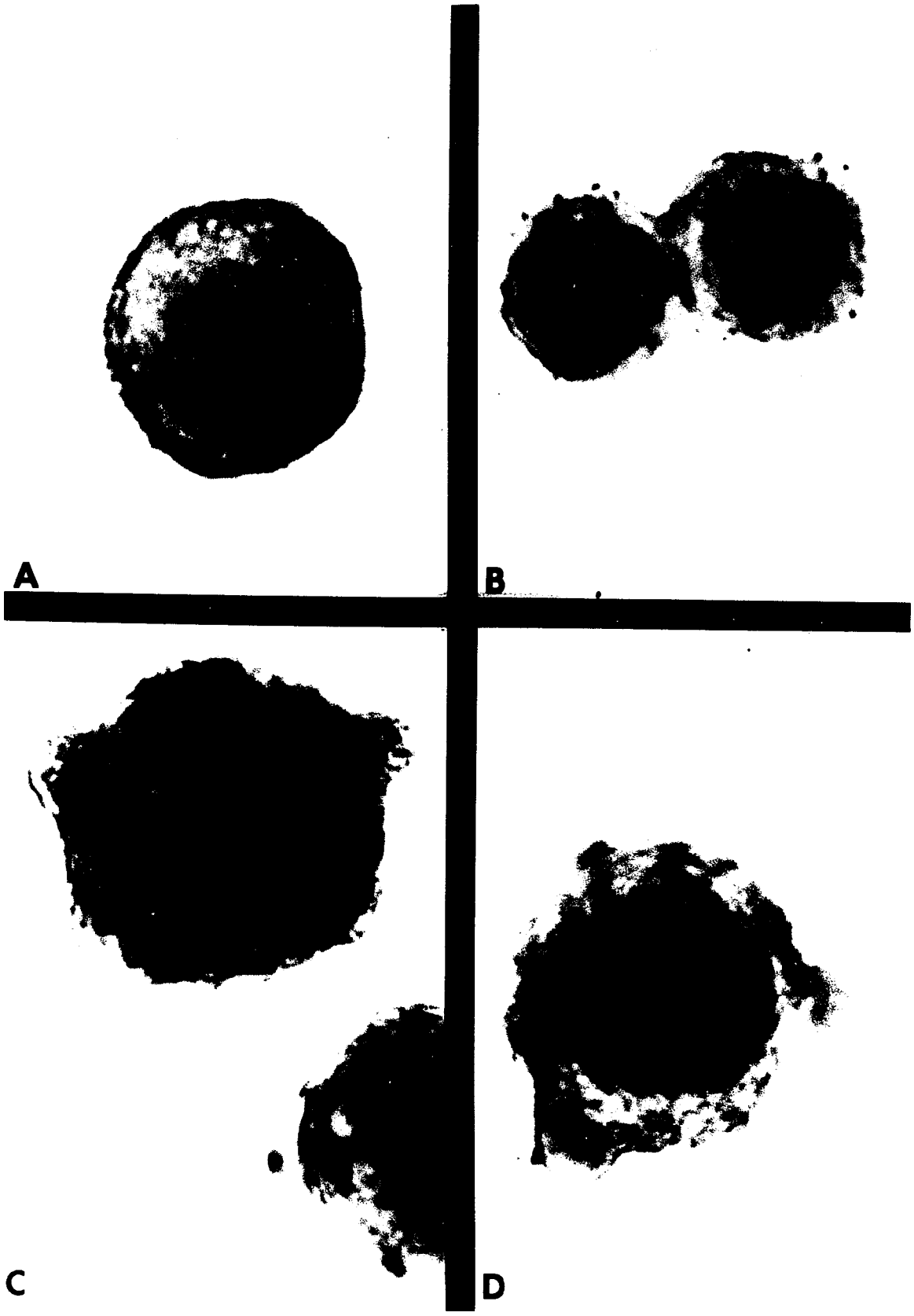
microscopic studies of hypotonically stressed cells. However, their cells were resuspended in isotonic medium before fixation and then suspended in a 300 mosm/kg buffer to which fixative had been added. In the current studies fewer vacuoles were seen if the gluteraldehyde was mixed in 98 mosm/kg buffer. The nuclear membranes were slightly separated in comparison to control cells.

The blebs contained numerous ribosomes normally found in the cytoplasm. No membrane separated the bleb contents from the remainder of the cell, although most of the organelles remained in the cytoplasm of the main body of the cell. The cell membrane and the bleb membrane were continuous (insert, figure 5B), but surface inflections were seen on the plasma membrane on either side of the bleb. These findings indicate that blebs are externally bounded by the plasma membrane, and that even though the blebs are continuous with the cytoplasmic compartment, there is a distinct discontinuity between the cytoplasmic ground substance and the interior of the bleb.

Surface Markers

Studies were carried out to determine whether bleb surface membranes were derived from existing cell membrane or whether they resulted from new membrane formation or the extrusion of intracellular membranes. If protein markers normally found on Ehrlich cell membranes were also present on the bleb surface, it would indicate the membrane used by the cell in the formation of the blebs had been present previously. Membrane markers were uniformly distributed over cell surfaces in both control and hypotonically stressed cells, with either anti IgG or anti ATPase plus anti IgG antibodies (figure 6). These markers were also distributed over bleb surfaces. The ATPase markers were found distributed similarly over positive controls (hog stomach). As expected, there were no membrane markers on the surfaces of the negative controls. The presence of these markers on bleb surfaces suggests that the membrane was

Figure 6. Immunocytochemical cell surface markers. 6a Anti-IgG. 6b Anti-ATPase. Magnification 1280X.


A
B
C
D

derived from pre-existing membrane; whether the membrane is of microvillous origin could not be determined.

CHANGES IN CELL VOLUME AND ION CONTENT

In order to measure the ion and volume changes which occurred within sixty minutes after hypotonic shock, twenty-three experiments were done and the changes observed are shown in figures 7 to 12. These figures and those that follow are graphs of the volume and ion changes observed during experimental manipulation. The volume changes are represented by the swelling index which is the ratio of intracellular water to cell dry weight. Intracellular ion content was expressed as micromoles per gram of dry weight of the cells.

The volume and ion changes for the first 240 seconds after mixing are graphed in figures 7 to 9. In control cells the swelling index varied slightly during the first four minutes. During this time K^+ content and Na^+ content also varied, but these variations were minor.

Figures 10 to 12 are graphs of longer time periods for which time is presented in minutes. The two enclosed areas represent the ninety-five percent confidence intervals of the results of these initial twenty-three experiments. The data points for the first four minutes were too numerous to be shown on these figures; however, they were included in the calculations of the ninety-five percent confidence intervals indicated by the enclosures. These confidence limits are included on all graphs of swelling index, K^+ content, and Na^+ content, and serve as a basis for comparison with results obtained when the ion content of the media was varied or inhibitors were added.

Ion and volume changes with hypotonic media were measured and correlated with observed and measured morphologic changes. The graphs in figures 7 and 10 depict the relative cell volume or swelling indices at different times in either control medium or in hypotonic medium. Figures 8 and 11 are

Figure 7. Short term effects of dilution on cell swelling index. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) to produce final osmolalities of 294 mosm/kg (isotonic) or 98 mosm/kg (hypotonically stressed).

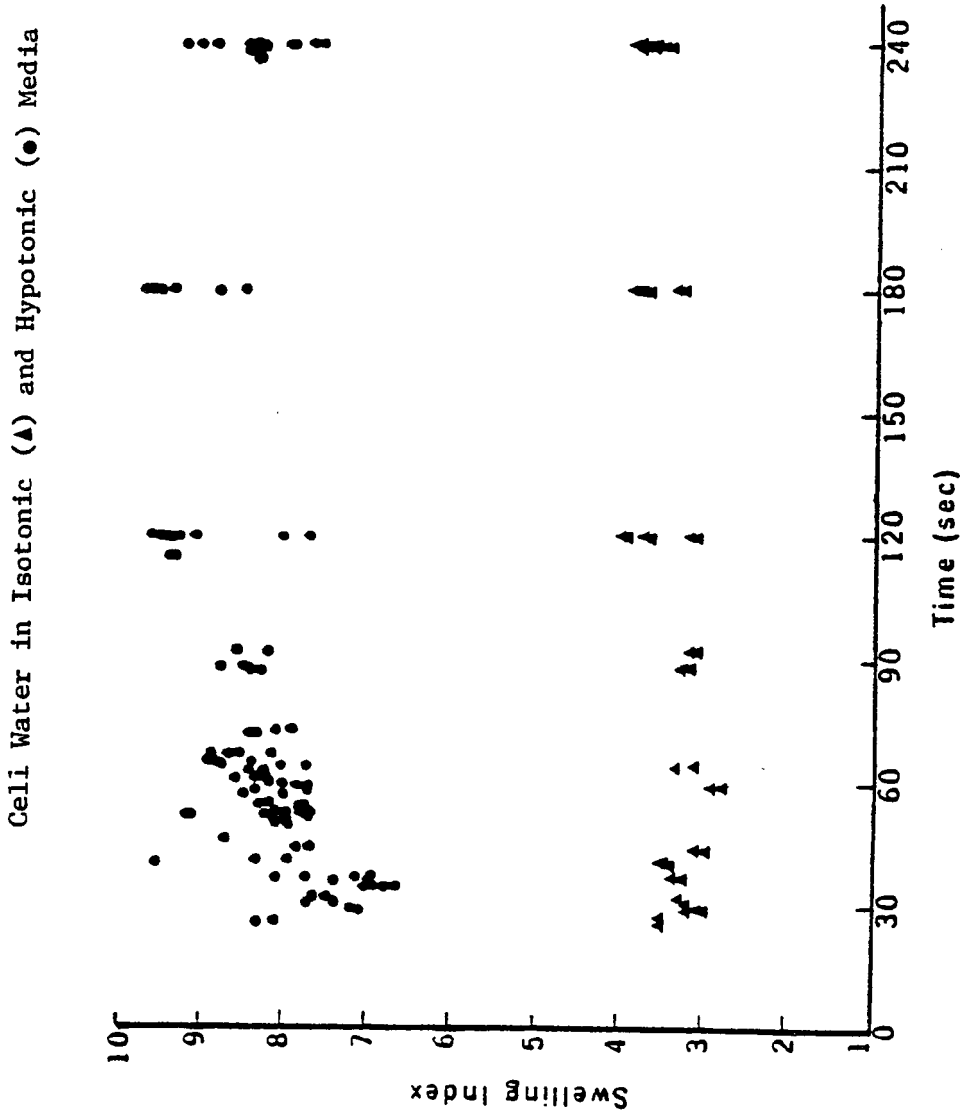

Cell Water in Isotonic (▲) and Hypotonic (●) Media
Swelling Index
Time (sec)
10
9
8
7
6
5
4
3
2
1
0
30
60
90
120
150
180
210
240

Figure 8. Short term effects of dilution on cell K^+. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) to produce final osmolalities of 294 mosm/kg (isotonic) or 98 mosm/kg (hypotonically stressed).

Cell Potassium Content in Isotonic (▲) and Hypotonic (●) Media

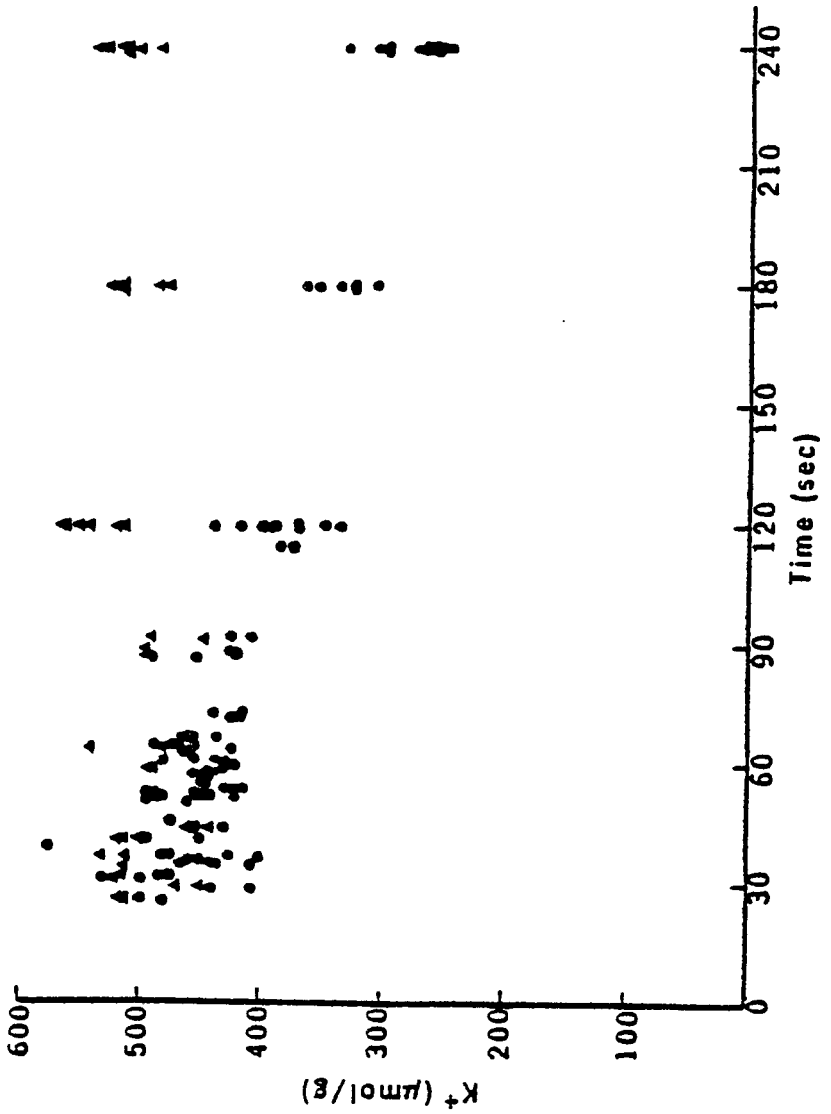


Figure 9. Short term effects of dilution on cell Na^+. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) to produce final osmolalities of 294 mosm/kg (isotonic) or 98 mosm/kg (hypotonically stressed).

Cell Sodium Content in Isotonic (▲) and Hypotonic (●) Media

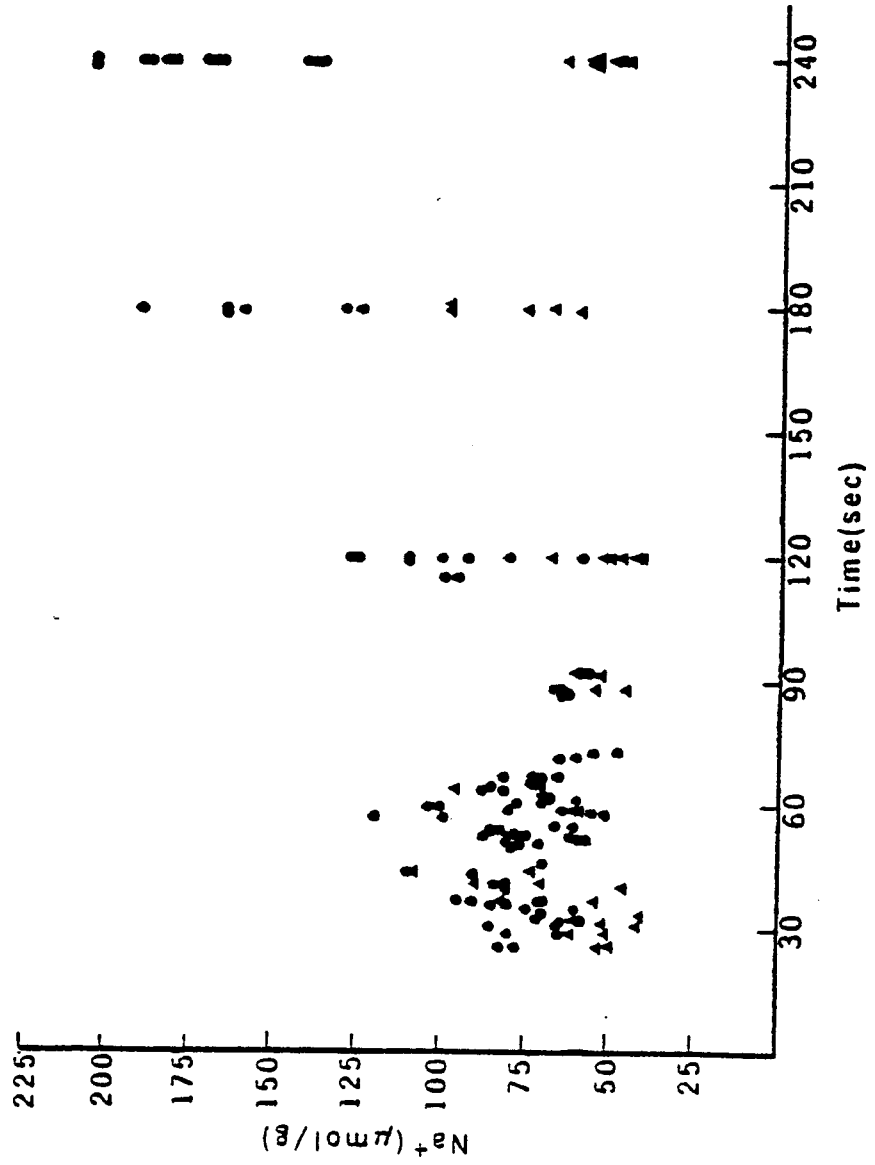


Figure 10. Long term effects of dilution on cell swelling index. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) to produce final osmolalities of 294 mosm/kg (isotonic) or 98 mosm/kg (hypotonically stressed). Solid lines represent the bounds of the 95% confidence limits.

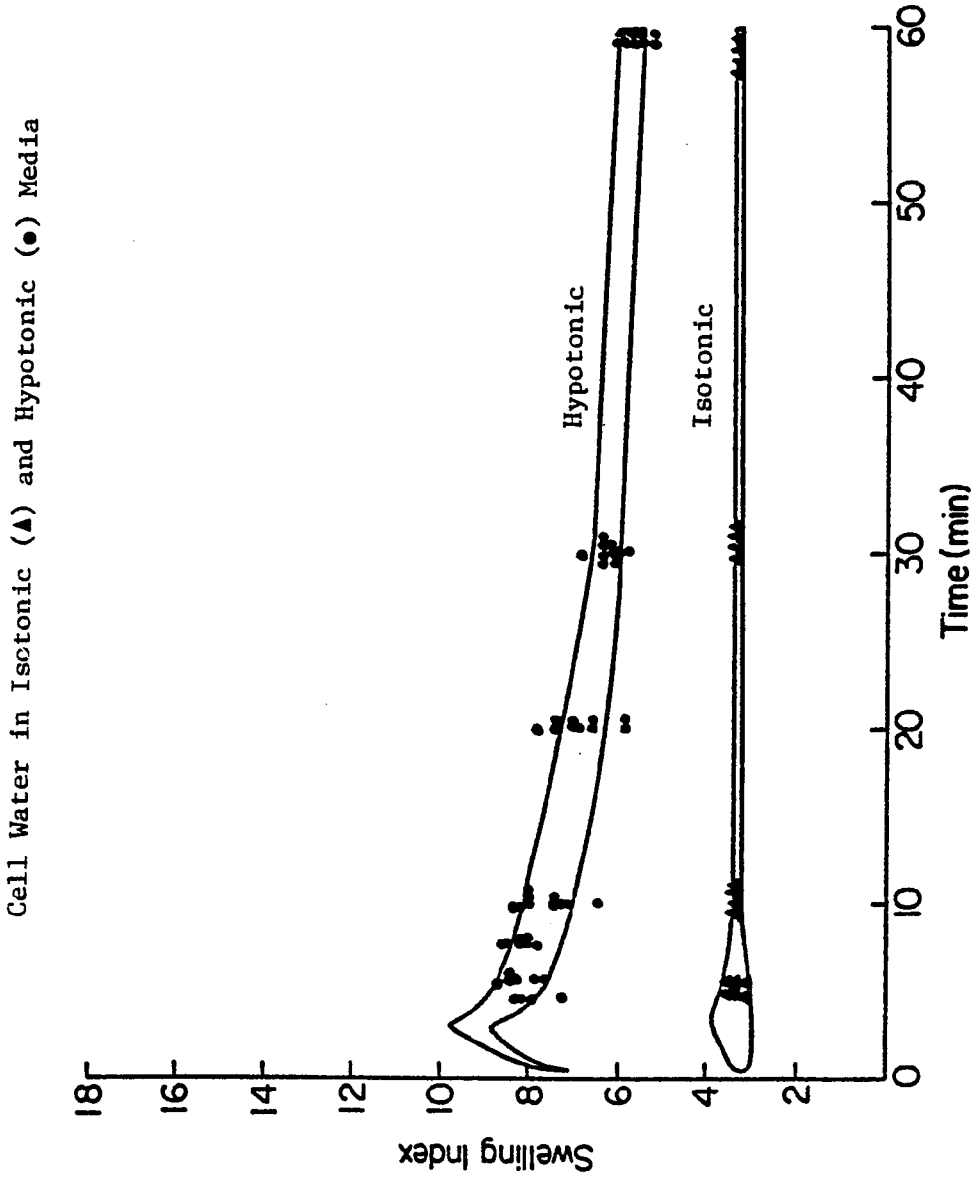

Cell Water in Isotonic (▲) and Hypotonic (●) Media
Swelling Index
18
16
14
12
10
8
6
4
2
0
10
20
30
40
50
60
Time (min)
Hypotonic
Isotonic

Figure 11. Long term effects of dilution on cell K^+. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) to produce final osmolalities of 294 mosm/kg (isotonic) or 98 mosm/kg (hypotonically stressed). Solid lines represent the bounds of the 95% confidence limits.

Cell Potassium Content in Isotonic (▲) and Hypotonic (●) Media

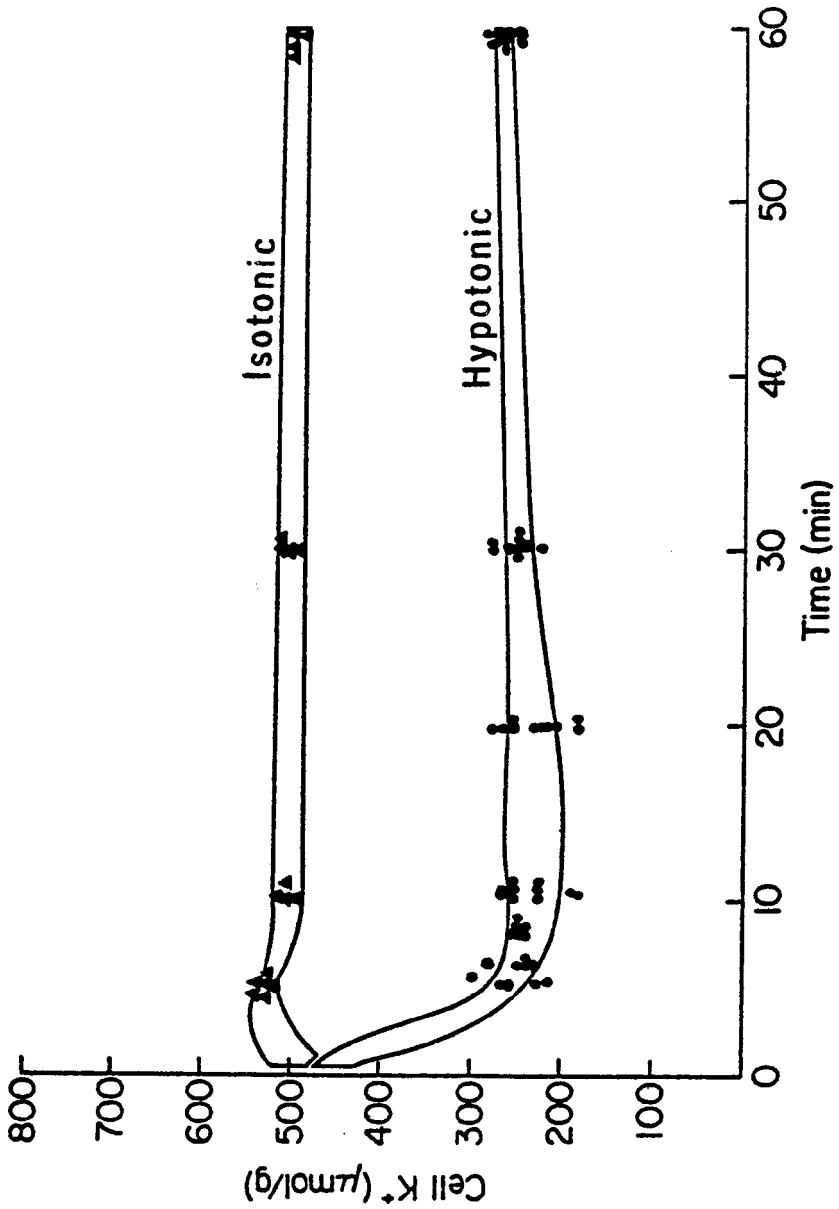
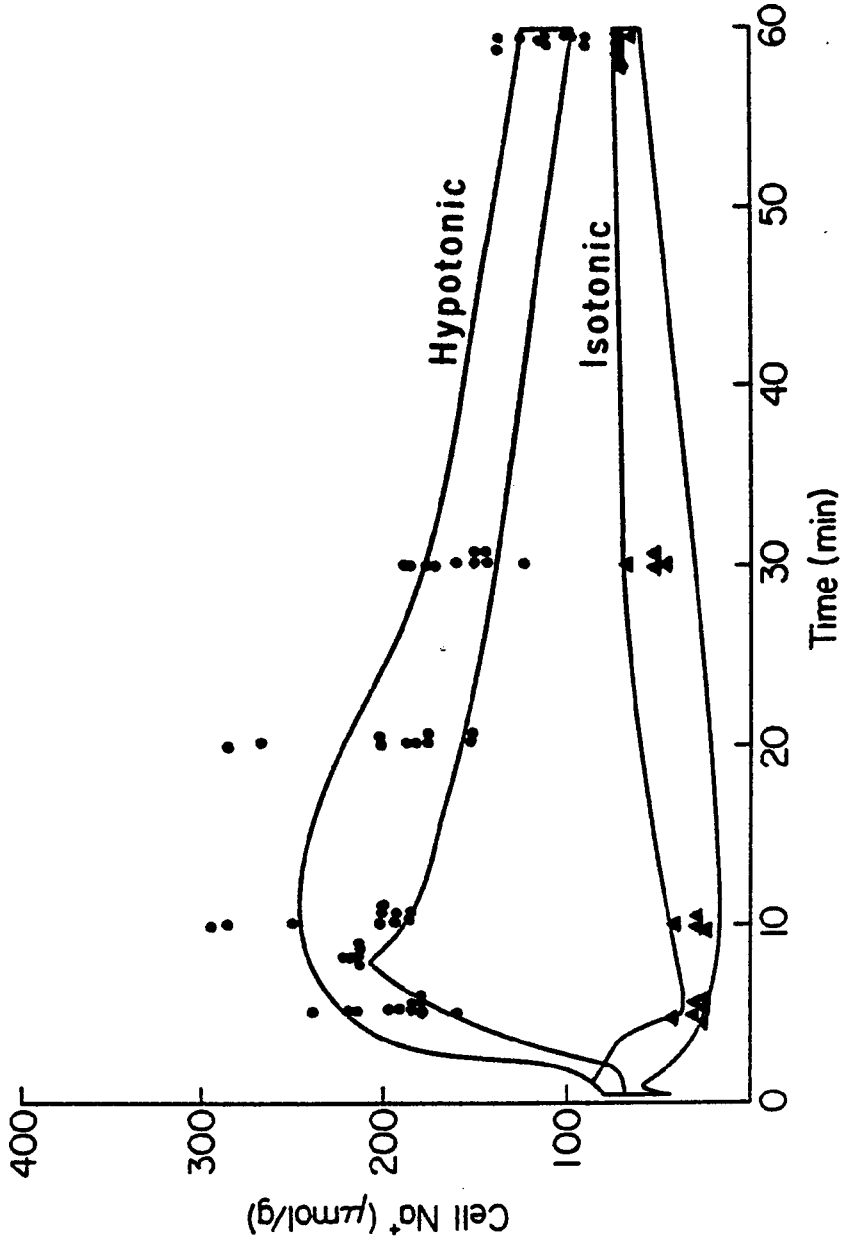


Figure 12. Long term effects of dilution on cell Na^+. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) to produce final osmolalities of 294 mosm/kg (isotonic) or 98 mosm/kg (hypotonically stressed). Solid lines represent the bounds of the 95% confidence limits.

Cell Sodium Content in Isotonic (▲) and Hypotonic (●) Media



graphs of the K^+ content of the cells and figures 9 and 12 depict the Na^+ content.

Swelling indices, and Na^+, K^+ and, in some cases Cl^- content were simultaneously measured in suspensions of control and hypotonically stressed cells. The control cells maintained relatively constant water and ion contents except for some slight initial scatter. In contrast, the swelling index of hypotonically stressed cells was significantly different at the first measurable time and remained higher than control cells throughout the hour of observation (figures 7 and 10). The fall of stressed cell K^+ and rise of Na^+ relative to control cells is described in detail below. Initial net K^+ and Na^+ fluxes were in the direction of their respective chemical gradients.

Control cell volumes and K^+ contents were constant after eight minutes; Na^+ contents varied longer and clustered more closely after thirty minutes. The slight decrease in control cell Na^+ at five minutes is unexplained but may be due to cell age and preincubation times as explained previously. Swelling indices at five and at thirty minutes were not significantly different from those at 0.4 to 0.6 of a minute, and K^+ and Na^+ contents at thirty minutes were not significantly different from those at 0.4 to 0.6 of a minute (table I).

The swelling indices for the hypotonically stressed cells were significantly different from the control cells at all measured times ($P < 0.001$). The Na^+ and K^+ contents were significantly different from controls after 120 seconds ($P < 0.001$ at 120, 180, and 240 seconds). Since the swelling indices were significantly different at thirty seconds, but significant ion content changes did not appear until 120 seconds, movement of water into the cell must occur first and alterations in ion content must follow.

There was a biphasic change in the volume of the stressed cells with a rapid two- to threefold increase during the first three minutes, followed by a

Table I

Swelling indices, Na^+, and K^+ contents at selected experimental time periods. Values represent mean ± S.E.M.

Parameter	Time	Control	Hypotonic	P
Swelling Index	0.4-0.6	3.26 ± 0.12	7.41 ± 0.24	
	5	3.32 ± 0.24	8.01 ± 0.34	0.01
	30	3.32 ± 0.05	6.32 ± 0.29	0.001
K^+	0.4-0.6	501.11 ± 19.72	458.82 ± 17.80	
	5	528.57 ± 10.53	248.82 ± 19.88	0.001
	30	504.37 ± 15.18	250.71 ± 14.44	0.001
Na^+	0.4-6.0	54.32 ± 9.05	74.38 ± 5.25	
	5	30.53 ± 6.46	198.13 ± 25.53	0.001
	30	51.65 ± 19.13	157.53 ± 16.95	0.001

Units: time = minutes, swelling index = μl/g, K^+ and Na^+ = μmol/g. P values indicate significance of difference from value at 0.4-0.6 minutes for hypotonic cells.

decline for the remaining fifty-seven minutes. A threefold increase in volume would be expected if the cells behaved as perfect osmometers. (Kregenow (1971) observed that duck erythrocytes also increased in volume only two-thirds as much as would be expected from perfect osmometers and attributed this difference partly to a loss of ions during the increase in volume.) The rate of decline was rapid initially but subsequently slowed. (This decline in volume while the cells were still suspended in hypotonic media is termed volume regulatory decrease.) Volume regulation in these cells was not complete; the swelling index did not return completely to baseline. The average swelling index for control cells at one minute was 3.12 while that for hypotonically stressed cells was 8.28, an increase of 165 percent. At thirty minutes the stressed cells had eliminated only thirty-eight percent of the increased volume.

To ensure that the volume decrease represented just a loss of fluid and not a loss of cells, intravital cell staining and cell dry weights per ml suspension were measured at the various time intervals. The two vital dyes used most commonly to indicate the presence of grossly altered membrane permeability for Erhlich tumor cells are nigrosin and trypan blue. Penttila and Trump (1975c) found no differences in the percentage of Ehrlich tumor cells stained with either of these two dyes, but trypan blue has been used with Erhlich ascites cells by many investigators (Mayhew and Levinson 1968, Penttila and Trump 1975c, Medzihradsky and Marks 1975) and was the dye chosen for these studies. Trypan blue diffusely stains the cytoplasm and nucleus of severely injured or dead cells (Pappenheimer 1917). The entrance of trypan blue into the cell is a strictly physical phenomenon based on the size of the dye particle; no chemical union between the dye and cytoplasm takes place (King et al. 1959b). The percentages of control cells stained in these experiments were 3.13 ± 0.33 (S.E.M.) percent at one minute and 4.07 ± 0.91 (S.E.M.) percent at thirty minutes. The percentages

of stressed cells stained were 4.34 $\pm$ 0.96 (S.E.M.) percent and 6.58 $\pm$ 1.48 (S.E.M.) percent at one and thirty minutes. None of the values were significantly different from the one minute control value. Other investigators found similar values; Mayhew and Levinson (1968) observed 2.5 percent, Medzihradsky and Marks (1975) observed less than 1.0 percent, and Penttila and Trump (1975a, 1975c) described 3.8 and 3.0 percent stained cells when observed up to three hours after harvesting. In addition, Laiho and Trump (1974) were unable to find any correlation between the number of stained cells and intracellular Na^+ content and volume.

If the volume decrease during volume regulation by stressed cells was caused by a loss of cell fragments as result of blebs being pinched off and discarded with the supernatant, this loss of cell mass would be evident as a decreased dry weight of centrifuged cells, because the fragments would be lighter than the cells and would not be centrifuged down into the cell pellet. Dry weights of control cells were compared with those of the hypotonically stressed cells (table II). No significant difference between the dry weights of the control cells was found at one and thirty minutes; however, a significant decrease from the one minute weight was present at sixty minutes. The weights of the stressed cells were significantly decreased from the one minute weights at both thirty and sixty minutes.

The decrease in cell dry weight occurred in both control and stressed cells, but the control cells maintained a constant swelling index. In addition, the loss of dry weight in the stressed cells was only approximately sixteen percent as large as the loss in volume. Therefore, the decrease in dry weight cannot account for the loss of cell volume.

Some possible causes of decreased cell weight in both stressed and control cells were disintegration and loss of cells, loss of intracellular particles,

Table II

Comparison of cell dry weights for either control or hypotonically stressed cells throughout the experimental procedure. Values represent the mean of the differences between cell dry weights at the time periods indicated.

Parameter	Time	Control	P	Hypotonic	P
Cell Dry Weight	30 vs 1	.86 ± .33	NS	1.24 ± .42	0.05
	60 vs 1	2.00 ± .48	0.05	1.24 ± .09	0.001

Units: time = minutes, weights = mg. P values indicate significance of difference between time periods indicated.

or loss of fluid-filled vesicles. Occasionally fragments of cell debris were noted during microscopy, and numerous lipid bodies were seen floating in the media. To determine whether the cells were being disrupted, cell suspensions were analyzed with density gradient centrifugation. During this analysis whole cells would be expected to form a pellet in the highest density sucrose, but cell fragments would be expected to be distributed throughout the gradient according to their individual densities.

Suspensions of cells harvested, washed, resuspended, and incubated as described, both control and stressed, were evaluated by density gradient centrifugation at one minute and at sixty minutes after dilution. Cells to be evaluated at sixty minutes were incubated in a shaking water bath prior to layering on the sucrose gradient.

Three different linear sucrose gradients were prepared, a ten to sixty percent (w/w) gradient, a ten to twenty percent (w/w) gradient and a twenty to sixty percent (w/w) gradient. Each gradient contained 7 ml of each sucrose soution with 1 ml of cell suspension carefully layered on top. Duplicate preparations were spun in a Beckman Model L5-65 ultracentrifuge at 26,000 x g for 7.5 hours. One-half ml aliquots were examined for sucrose density in an Abbé refractometer and for protein in a Gilford 250 spectrophotometer (280 nm). The gradients were linear in all experiments. Negligible amounts of protein (less than ten percent of the total present) were found at the level of least density. Less than three percent protein was found at any other levels except the most dense level where the whole cells had formed a pellet. These results indicated that little cell protein could have been lost in the form of cell debris or as pinched-off blebs. Lange (1964) also found that volume regulation in response to diluted media was associated with concomitant reductions in weight, and Macknight and Leaf (1977) observed that losses of water paralleled any loss of

solid matter so that any changes in cell volume should be reflected by cell water content per unit cell dry weight.

Control cell Na^+ content varied slightly during the first five minutes, falling at first but recovering and becoming relatively constant thereafter. The Na^+ content of hypotonically stressed cells rose rapidly to a peak at approximately eight minutes and steadily decreased thereafter. Changes in the Na^+ content of the stressed cells did not parallel the swelling index but initially lagged slightly behind (figures 10 and 12) (perhaps because the plasma membrane is highly permeable to water, but passive salt movement is restricted). Initial control cell Na^+ content ranged from 40 to 85 μmol/g and the final content was 60 to 70 μmol/g. Stressed cell Na^+ content was 50 to 100 μmol/g initially and 100 to 130 μmol/g at one hour.

After some initial scatter, the control cell K^+ was relatively constant. Control K^+ content was initially 450 to 540 μmol/g with final values of 490 to 510 μmol/g. In contrast the K^+ content of the hypotonically stressed cells fell rapidly for seven to ten minutes and then rose slowly between twenty and sixty minutes. The stressed cell K^+ content was 410 to 530 μmol/g initially and 240 to 290 μmol/g at one hour. Thus, potassium loss from stressed cells was greater than Na^+ gain. The difference between the initial four-tenths to six-tenths of a minute values and all later values for stressed cell swelling index, K^+ content, and Na^+ content were statistically significant (table I).

In summary, the Ehrlich tumor cells did swell initially when placed in hypotonic media, but were able to begin regulating their volume after about three minutes while still suspended in the same medium. Prior to and during the initial volume regulatory decrease, the cell Na^+ content increased, but then steadily declined. Over the same time period, the K^+ content first decreased then much more slowly increased.

MORPHOMETRIC DETERMINATION OF CELL VOLUME CHANGES

By morphometric analyses with Nomarski microscopy the contribution of blebs to the increased cell volumes observed in the initial rapid swelling phase could be determined (Delesse 1847). The primary observations are detailed below. When the cells were suspended in hypotonic media, bleb formation could account for most of the volume increase, and bleb resorption appeared to account for the volume regulatory decrease.

The number of cells per suspension were determined with a Coulter counter. The percentage of these cells that were permeable to vital dyes and thus not considered viable was 3.1 and the cell numbers determined with the Coulter counter were corrected for these non viable cells. Both intracellular water and dry weights were known for all experiments. Using these parameters and correcting the cell dry weight for density, approximate cell volumes were calculated (table III). These volumes were obtained by dividing the intracellular water plus corrected cell dry weight by the number of viable cells.

As shown by Chayes (1965), sampling was not simply random, but was systematic with a random start, because once the first point on the coverslip was chosen, all of the other points on that coverslip were fixed in that the cells were all part of the drop of suspension chosen at that time. The boundaries of the coverslip imposed a relationship among cells within those boundaries and excluded any measurements outside them. However, this study was designed so that a high degree of accuracy on a limited number of points was not necessary. Instead, the average of many measurements of structures and their components was needed. In this case unbiased sampling required selection of a few fields from many coverslip preparations (Weibel 1963). As discussed in methods, subsampling was as random as possible.

Table III

Comparison of isotonic cell volumes measured from cell weights and by morphometry. Values represent mean ± S.E.M.

Methods	Volume	n
Cell Weight	1,349 ± 12	33
Morphometry		
Spheriod	2,040 ± 26	191
Prolate Ellipsoid	1,711 ± 22	191
Oblate Ellipsoid	2,435 ± 30	191

Units: volume = μm^3.

Table IV

Areas of isotonic and hypotonically stressed cells at one and thirty minutes. Values represent mean ± S.D.

Group	Time	Area	n	P
Isotonic	1	191.0 ± 52.6	191	
	30	168.2 ± 28.6	93	
Hypotonic				
Total	1	297.7 ± 81.8	81	0.001
Without Blebs		203.2 ± 58.0	81	NS
Total	30	237.7 ± 72.5	113	0.05

Units: time = minutes, area = μ^2. P values indicate significance of difference from control cells at identical time periods.

Sixteen experiments were carried out with both control and hypotonically stressed cells. Five to forty profiles were included in each sample in order to obtain reliable averages for areas and perimeters. Cells chosen as randomly as possible were measured and the fractional circularity was calculated. Fractional circularity is a percentage comparison of the area of a circle (with a circumference identical to the perimeter of the cell) to the measured area of the cell. Cell perimeters were drawn at one and thirty minutes after dilution; perimeters and areas were measured by computer (table IV).

At one minute the control cell area was 191.0 $\pm$ 3.8 μ^2 (S.E.M.) while the stressed cell area was 297.7 $\pm$ 9.1 μ^2 (S.E.M.), an increase of fifty-six percent. By thirty minutes the stressed cell area had decreased to 237.7 $\pm$ 6.8 μ^2 (S.E.M.), indicating that fifty-six percent of the cell area increase had been eliminated. During this same time period approximately thirty-eight percent of the volume increase had been eliminated. Neither the cell area nor volume was returned to baseline levels by the cell volume adjustment. The areas of the control cells (191.0 μ^2) were not significantly different from the areas of stressed cells (203.2 μ^2) defined by the cytoplasmic matrix, indicating that the increase in cell area could be accounted for by the bleb area. The area to volume ratio of the control cells was 0.1415 while that of the stressed cells was 0.1033 at one and thirty minutes. The absence of change in the area to volume ratio of the stressed cells indicates that both parameters were diminishing at approximately the same rate. By thirty minutes, protruding blebs (figure 3C) were no longer visible. The excess volume surrounded the cytoplasm of the cells, causing the cell outline to appear round in spite of the long, thin microvilli becoming evident along the cell surface.

Fractional circularity can be determined by the formula:

$$\text{fractional circularity} = (\text{true area})(4\pi)/(\text{perimeter})^2$$

The circularity of a circle is one. All other shapes have a circularity of less than one; a square is approximately 0.79. The mean circularity of these cells was 0.939, indicating that they might be slightly ellipsoid. However, since visual determination of whether the cells were ellipsoid or spheriod in shape was difficult, efforts were made to determine their true shape by computing their volumes after assuming they were either spherical or ellipsoidal.

The volume of a sphere is $V = (4/3)\pi r^3$. The radius, r, was calculated from the area measured with Nomarski optics, assuming the area was a circle.

The volume of prolate ellipsoids were calculated as:

$$V = (4/3)\pi a b^2$$

The volume of oblate ellipsoids were determined by the formula:

$$V = (4/3)\pi a^2 b$$

For these calculations the circumference was assumed to be approximately equal to $(2\pi)((a^2 + b^2)/2)^{1/2}$ with "a" as the major axis and "b" as the minor axis calculated by the formulas:

$$a = \text{area}/\pi b$$

$$b = ((\text{perimeter}^2 + (\text{perimeter}^4 - 16\pi^2 \text{area}^2)1/2)/4\pi^2)1/2$$

The directly measured volume most closely approximated the calculated volume when the cell shape was assumed to be a prolate ellipsoid (table III).

EFFECT OF INHIBITORS

Since the formation of blebs and volume regulation were apparently related, preliminary studies of several cell function inhibitors which alter either bleb formation or volume control in other systems were carried out. In some studies the medium was changed. In the Na^+ free experiments, choline chloride (choline-KRP), KCl (K-KRP), or LiCl (Li-KRP) were substitued for NaCl in equiosmolar amounts and the sodium phosphate buffer was replaced with a potassium phosphate buffer. For K^+ free experiments, NaCl was substituted for

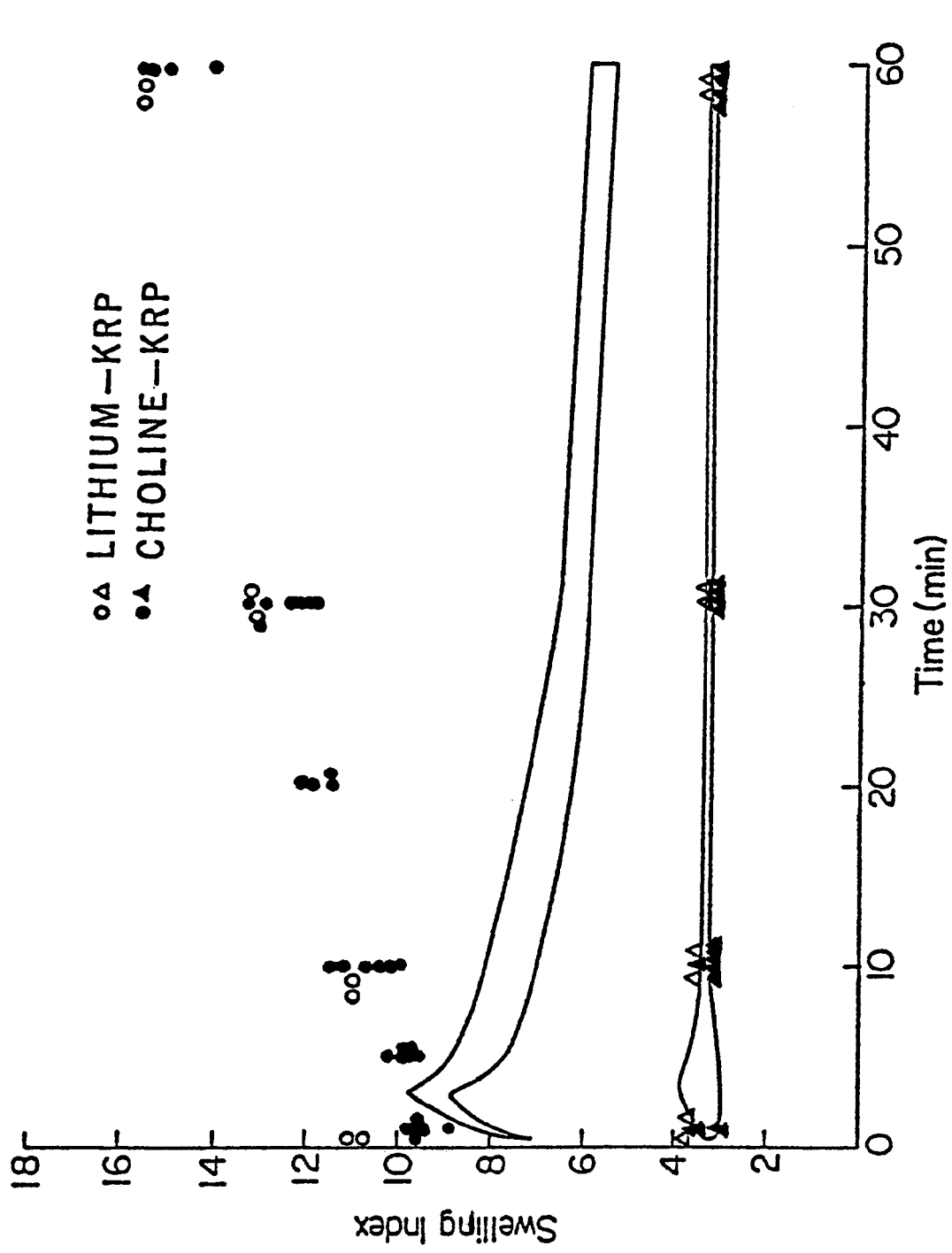
KCl (K^+ free-KRP). For Cl^- free experiments, sodium methylsulfate or $NaNO_3$ were combined with K_2SO_4 as Cl^- replacements.

In other experiments the inhibitors furosemide, ethacrynic acid, colchicine, amiloride, ouabain, and dibutyryl cAMP were added. Experimental data were plotted on graphs on which the ninety-five percent confidence intervals for the studies with Na-KRP media had previously been inscribed. If the medium change or inhibitor caused the swelling index or ion contents to fall outside of these ninety-five percent confidence limits, the data were accepted as significant.

Initially the effect of diluting all of the ions to an equal extent was determined by diluting the Na-KRP buffer with distilled water. Subsequently the cells were placed in a solution in which the osmolality had been reduced to 98 mosm/kg by reducing only NaCl. (The other ions had not been diluted.) No differences were found. Swelling indices and Na^+ and K^+ contents in medium in which only NaCl was decreased all fell within the ninety-five percent confidence limits at all time points. These findings indicated that bleb formation was related only to the osmolality or a decrease in NaCl.

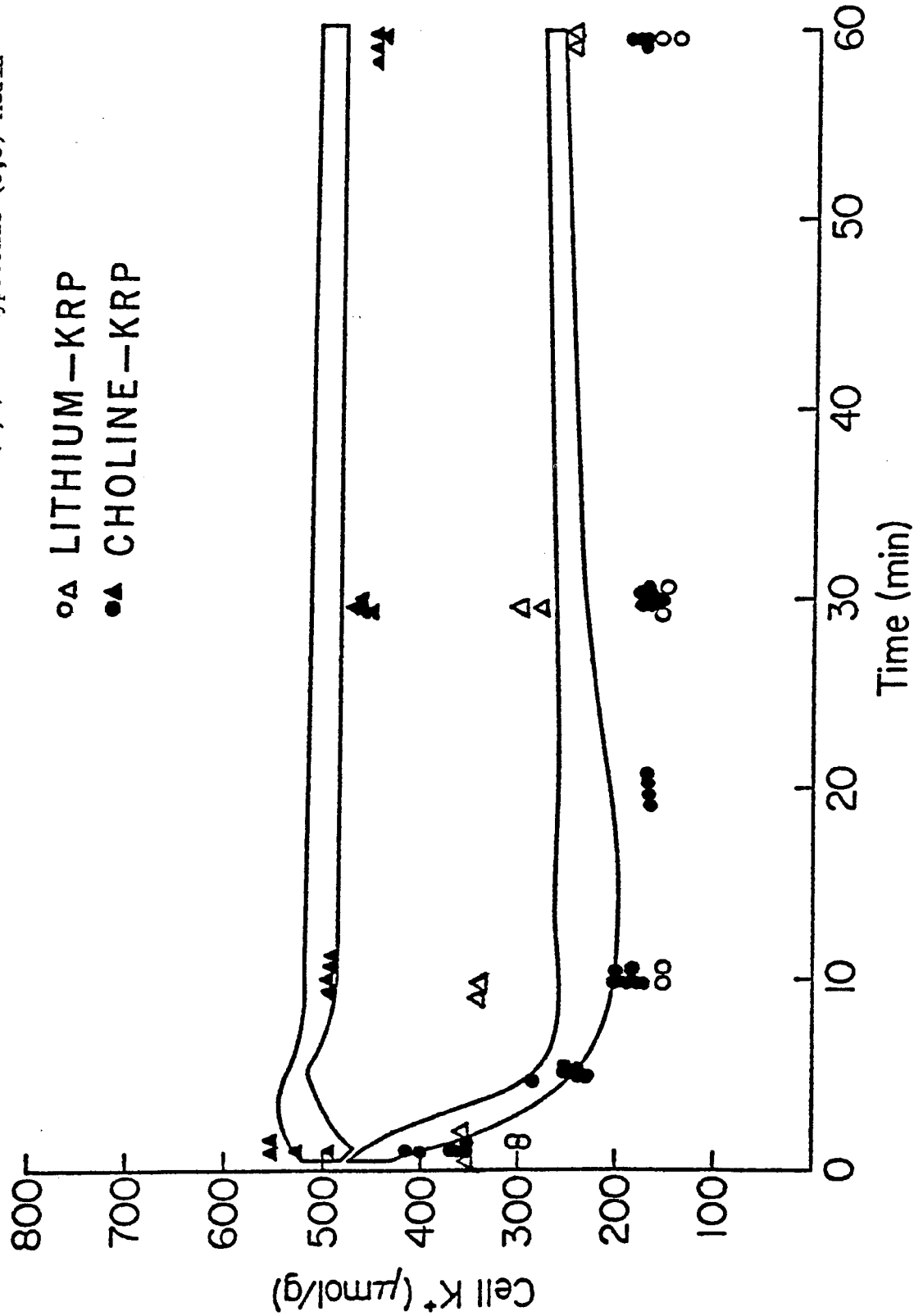
To test the effects of Na^+ exclusion, choline-KRP was used in three experiments. Cell membranes are relatively impermeable to choline; its substitution would be expected to result in a decrease in cell volume. The control cells were slightly shrunken after thirty minutes, but the stressed cells were far more swollen than stressed cells incubated in Na-KRP. As illustrated in figure 13, the swelling index rose continuously from mixing to sixty minutes. Water has entered these cells and possibly some choline also. Sodium content is not shown because Na^+ left the cells as expected, and the Na^+ content fell to less than 25 μmol/g and remained constant in both control and stressed cells. Cell K^+ content is depicted in figure 14. Both control and stressed cell K^+

Figure 13. Effect of Na^+ replacement on cell swelling index. At time zero cell suspensions which had been preincubated in choline-KRP were diluted with either isotonic choline-KRP (closed triangles) or water (closed circles) or cell suspensions which had been preincubated in Li-KRP were diluted with either isotonic Li-KRP (open triangles) or water (open circles). Swelling indices in the absence of Na^+ are compared to the 95% confidence bounds (solid lines) derived for control cells (with Na^+, figure 10).



Effect of Sodium Replacement on Cell Water in Isotonic (▲,△) and Hypotonic (●,○) Media

Figure 14. Effect of Na^+ replacement on cell K^+ content. At zero time cell suspensions which had been preincubated in choline-KRP were diluted with either isotonic choline-KRP (closed triangles) or water (closed circles) or cell suspensions which had been preincubated in Li-KRP were dilutd with either isotonic Li-KRP (open triangles) or water (open circles). Values for potassium content in the absence of Na^+ are compared to the 95% confidence bounds (solid lines) derived for control cells (with Na^+, figure 11).



Effect of Sodium Replacement on Cell Potassium in Isotonic (▲,Δ) and Hypotonic (●,○) Media

content decreased with time. The cells appeared to lose almost all of their Na^+ and more K^+ than cells suspended in Na-KRP. The total loss of Na^+ plus K^+ in both the hypotonically stressed and control choline treated cells was greater than that seen in Na-KRP treated stressed or control cells. In order to make certain that it was the Na^+ removal that abolished the volume regulatory decrease, additional studies were done with media in which NaCl was replaced with LiCl and the sodium buffer was replaced with a potassium buffer. The results were similar (figures 13 and 14, the Na^+ content is not shown because Na^+ levels were constant and just above zero). In human erythrocytes, lithium replaces Na^+ in the Na^+/Na^+ exchange system and the Na^+/K^+ cotransport system and intracellular Li^+ can be transported on the Na^+/K^+ pump to promote K^+ influx if Na^+ is absent intracellularly (Canessa et al. 1982). Potassium replacement of Na^+ (not shown) was also found to inhibit volume regulation. These results imply that extracellular sodium is necessary for volume control and that the regulation of Na^+ movement may be essential for volume regulation.

Since extracellular Cl^- levels could have influenced the volume regulatory decrease, two different Cl^- free media were used in eleven experiments. The K^+ as KCl in each medium was replaced with identical amounts of K^+ as K_2SO_4, and the NaCl was replaced with either $NaNO_3$ or sodium methylsulfate. The swelling index for control cells decreased slightly with the sodium methylsulfate; no effect on the volume regulatory decrease could be found with either medium. The Cl^- content of the control cells decreased slightly from 123 to 100 μmol/g in the NO_3^- media and was unchanged in the methylsulfate media. The stressed cell Cl^- decreased from 132 to 124 μmol/g with NO_3^- and was unchanged with methylsulfate.

Although dilution of K^+ had no detectable effect on the volume regulatory decrease, it appeared that its total exclusion might, since both K^+ and

norepinephrine alter volume control in erythrocytes (Kregenow 1981) and since Rosenberg and his coworkers (1972) have shown that K^+ free medium inhibited the volume regulatory decrease in Ehrlich cells. In three experiments K^+ free medium was prepared by replacing the KCl in the Na-KRP buffer with osmotically equivalent NaCl. No alteration occurred in the swelling index for the stressed cells except for a decrease slightly below the ninety-five percent confidence interval calculated for cells in Na-KRP buffer at sixty minutes. The stressed cell K^+ decreased only slightly and the Na^+ increased slightly. The control cells initially shrank and remained at the lower volume; they had a larger loss of K^+ and a larger increase in Na^+ than stressed cells.

Electrolyte transport and intracellular composition of some cells may be partially regulated by catecholamine induced changes in intracellular cAMP (Riddick et al. 1971) and dibutyryl cAMP mimics these catecholamine effects on electrolyte transport in liver, renal, and some tumor cells (Riddick et al. 1971). It has been observed that Ehrlich cell membrane preparations can form cAMP (Wikstrom et al. 1971). Additionally, Panniers and Clemens (1981) found that dibutyryl cAMP crosses the Ehrlich cell membrane; therefore, dibutyryl cAMP in concentrations of 1.0 mM or 0.001 mM was added to the sidearm solutions prior to incubation in two studies. Widely different concentrations were used so that the higher concentration would overwhelm the tendency for cAMP to be compartmentalized around the cell membrane. When swelling indices, Na^+ content, and K^+ content were compared with the results obtained in Na-KRP, only the Na^+ content fell outside of the ninety-five percent confidence intervals. It was slightly increased in both control and stressed cells. The addition of dibutyryl cAMP had no effect on the volume regulation of hypotonic cells.

Amiloride is a K^+ sparing diuretic which has been used to elucidate the molecular basis and physiological regulation of diffusional and of facilitated Na^+

entry into tissues and cells. Amiloride is thought to reversibly block Na^+ transport which is part of a Na^+/H^+ electroneutral exchange system found in some cells. This system appears to be involved in both volume regulation and in intracellular pH regulation, but is postulated to be operational only after external stimulation. Volume regulatory systems sensitive to amiloride have relatively low affinities for the drug, and high concentraitons are necessary for inhibition (Benos 1982). The effects of various concentrations of amiloride were explored in three experiments (figures 15 to 17). No changes in the volume regulatory decrease or control cell volume could be found. After hypotonic stress in the presence of amiloride, cellular Na^+ underwent an exaggerated increase and the K^+ had a similar decrease. Since it was still uncertain that high enough concentrations of the drug were used and since other investigators have found that acidic pH has a protective effect on cell ultrastructure during stress, the effects of high H^+ ion concentrations were tested in one experiment. The pH of the medium in this experiment was reduced to 6.85, but no changes in the responses of either control or stressed cells were found.

Since important cellular changes during the volume regulatory phase involved both Na^+ and K^+, cells were incubated with the cardiac glycoside ouabain, an agent that has been shown to reversibly inhibit net Na^+/K^+ transport in cultured Ehrlich cells (Mayhew and Levinson 1968). Mayhew and Levinson (1968) have also observed that blebs develop on these cells after several hours of incubation in ouabain containing media. Ouabain in a concentration of 0.1 mM was added to the sidearm solutions prior to incubation in four experiments. The volume regulatory decrease was unaffected, but the swelling index of control cells was slightly increased (figure 18). This increase could possibly be accounted for by a slight increase in Na^+ (figures 19 and 20). In stressed cells intracellular Na^+ rose and K^+ fell.

Figure 15. Effect of amiloride on cell swelling index. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles, diamonds, and squares) containing amiloride to produce the final suspension concentrations indicated. Cell swelling index in the presence of amiloride is compared to the 95% confidence bounds (solid lines) derived for control cells (without amiloride, figure 10).

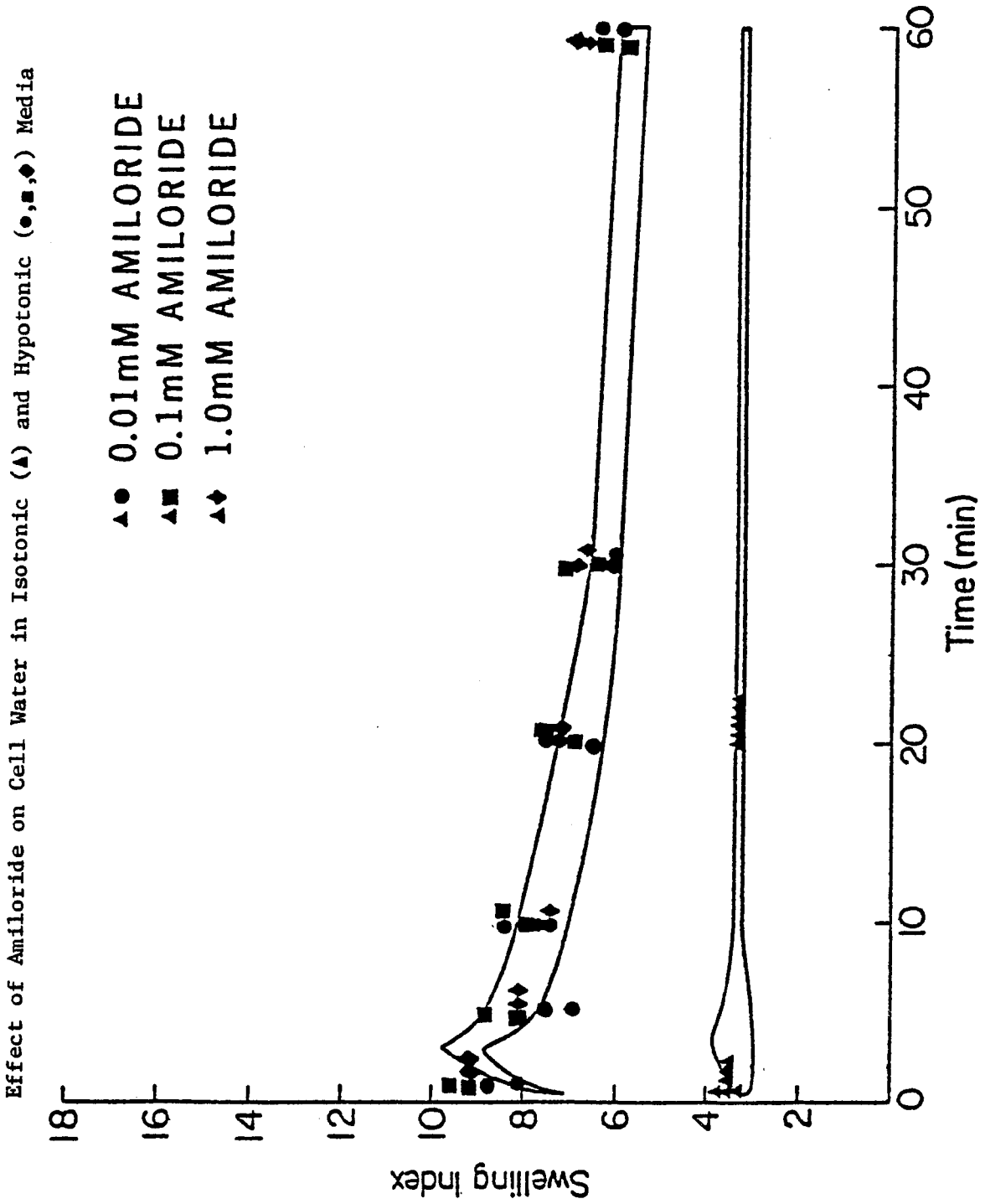

Effect of Amiloride on Cell Water in Isotonic (▲) and Hypotonic (●,■,◆) Media
▲● 0.01mM AMILORIDE
▲■ 0.1mM AMILORIDE
▲◆ 1.0mM AMILORIDE
Swelling Index
Time (min)
18
16
14
12
10
8
6
4
2
0
10
20
30
40
50
60

Figure 16. Effect of amiloride on cell K^+. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles, diamonds, and squares) containing amiloride to produce the final suspension concentrations indicated. Cell K^+ content in the presence of amiloride is compared to the 95% confidence bounds (solid lines) derived for control cells (without amiloride, figure 11).

Effect of Amiloride on Cell Potassium in Isotonic (▲) and Hypotonic (●,■,◆) Media

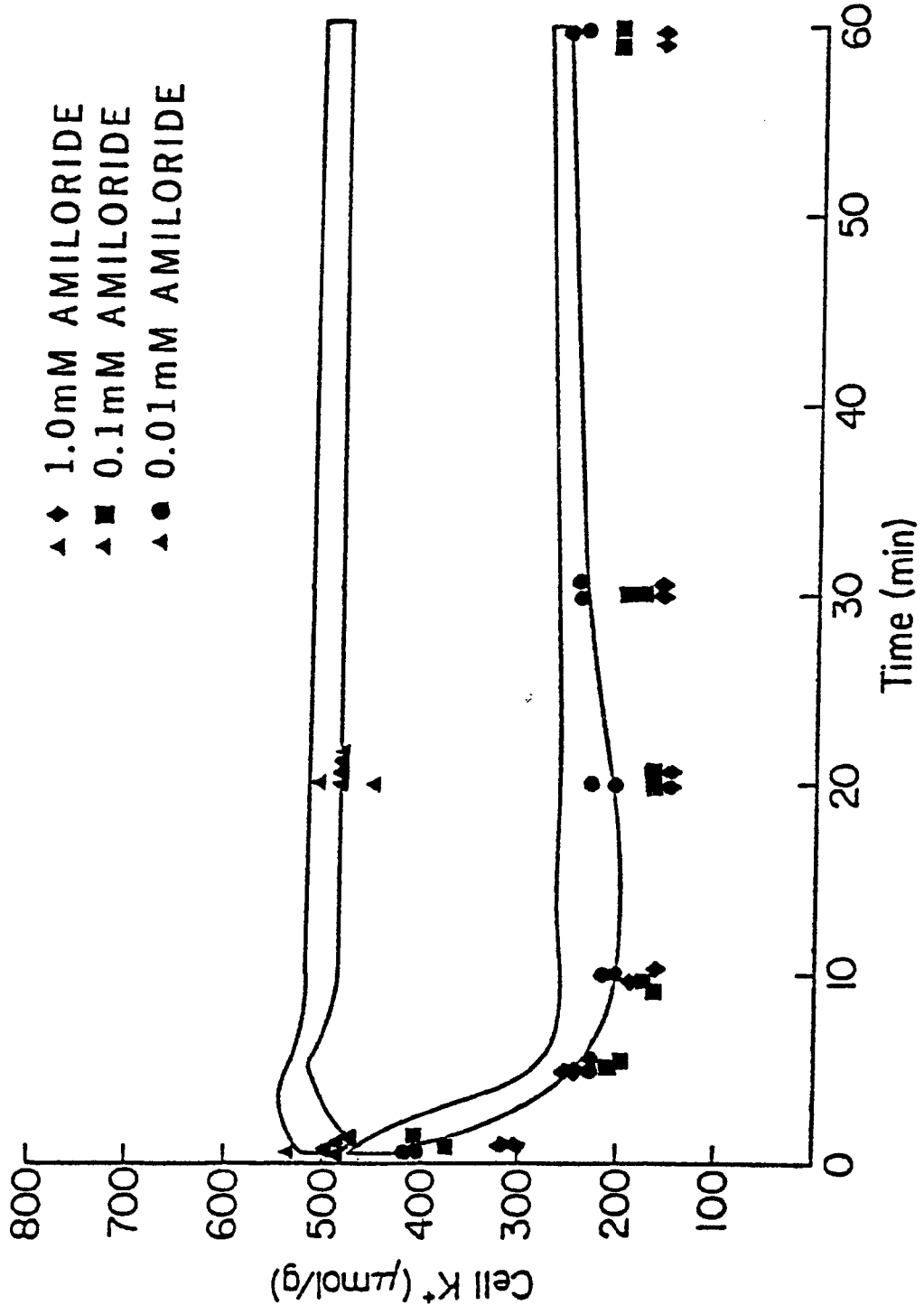


Figure 17. Effect of amiloride on cell Na^+. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles, diamonds, and squares) containing amiloride to produce the final suspension concentrations indicated. Cell Na^+ content in the presence of amiloride is compared to the 95% confidence bounds (solid lines) derived for control cells (without amiloride, figure 12).

Effect of Amiloride on Cell Sodium in Isotonic (▲) and Hypotonic (●,■,◆) Media

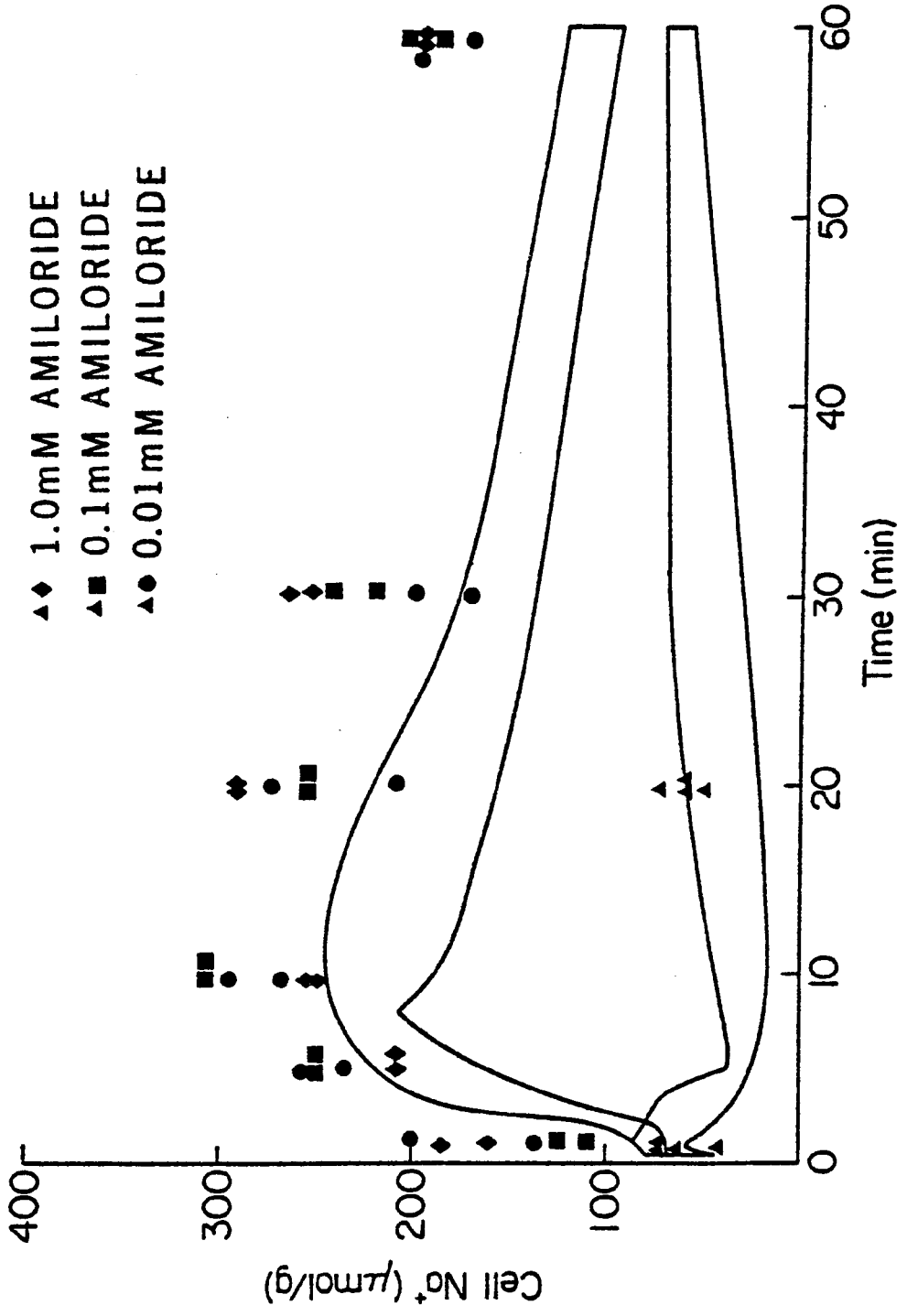


Figure 18. Effect of ouabain on cell swelling index. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) containing ouabain to produce the final suspension concentration of 0.1 mM. Cell swelling index in the presence of ouabain is compared to the 95% confidence bounds (solid lines) derived for control cells (without ouabain, figure 10).

Effect of Ouabain on Cell Water in Isotonic (▲) and Hypotonic (●) Media

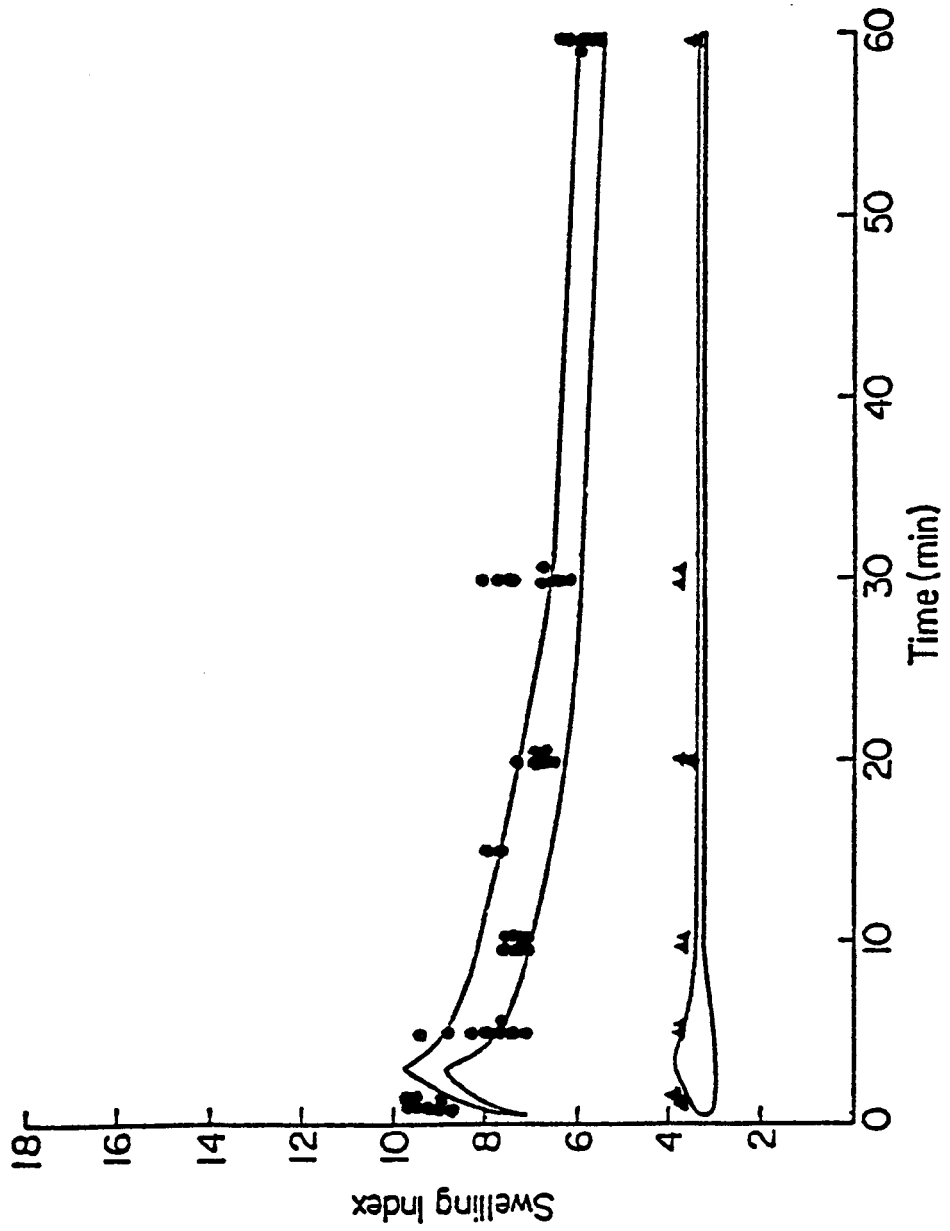


Figure 19. Effect of ouabain on cell K^+. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) containing ouabain to produce the final suspension concentration of 0.1 mM. Cell K^+ content in the presence of ouabain is compared to the 95% confidence bounds (solid lines) derived for control cells (without ouabain, figure 11).

Effect of Ouabain on Cell Potassium in Isotonic (▲) and Hypotonic (●) Media

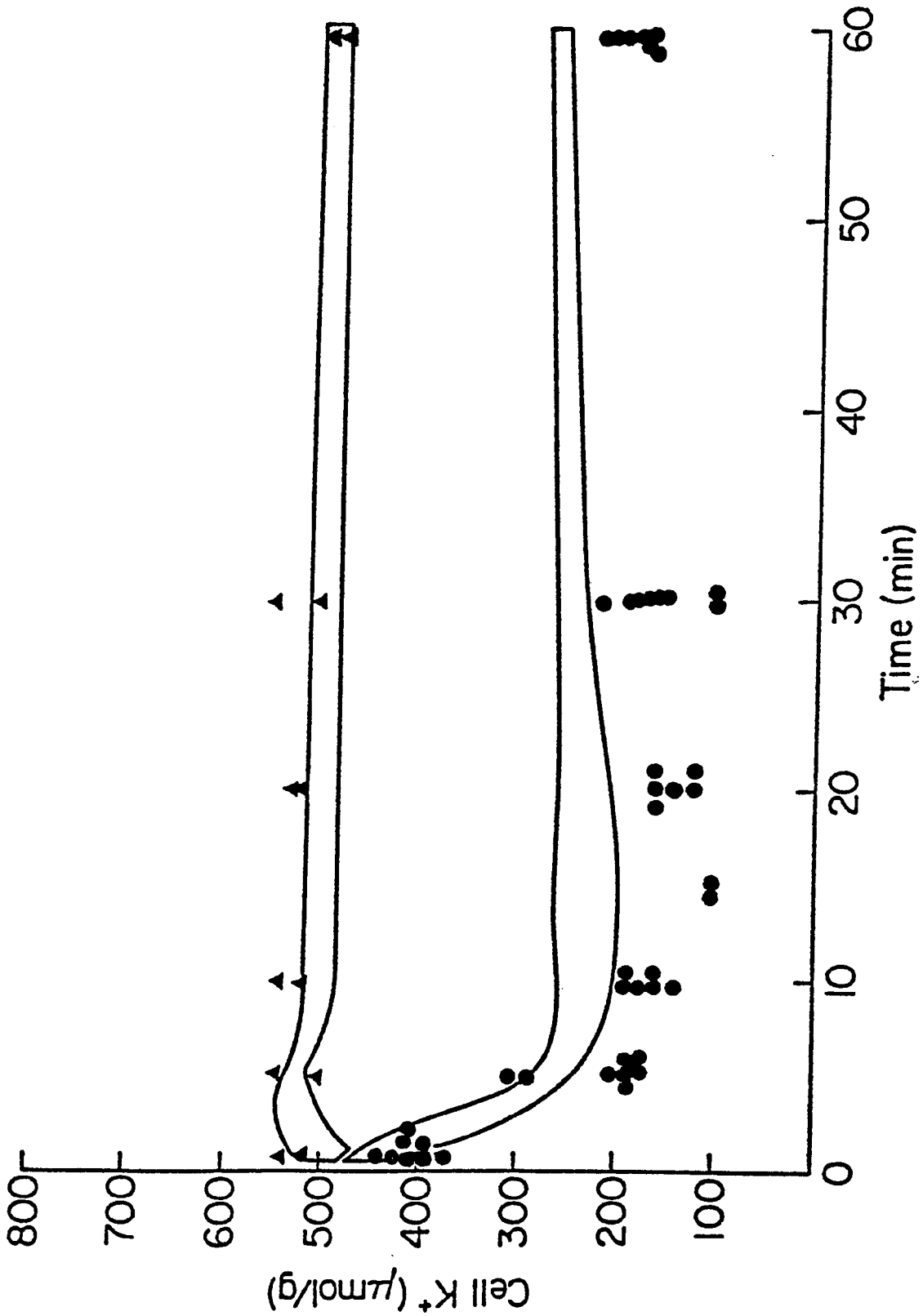
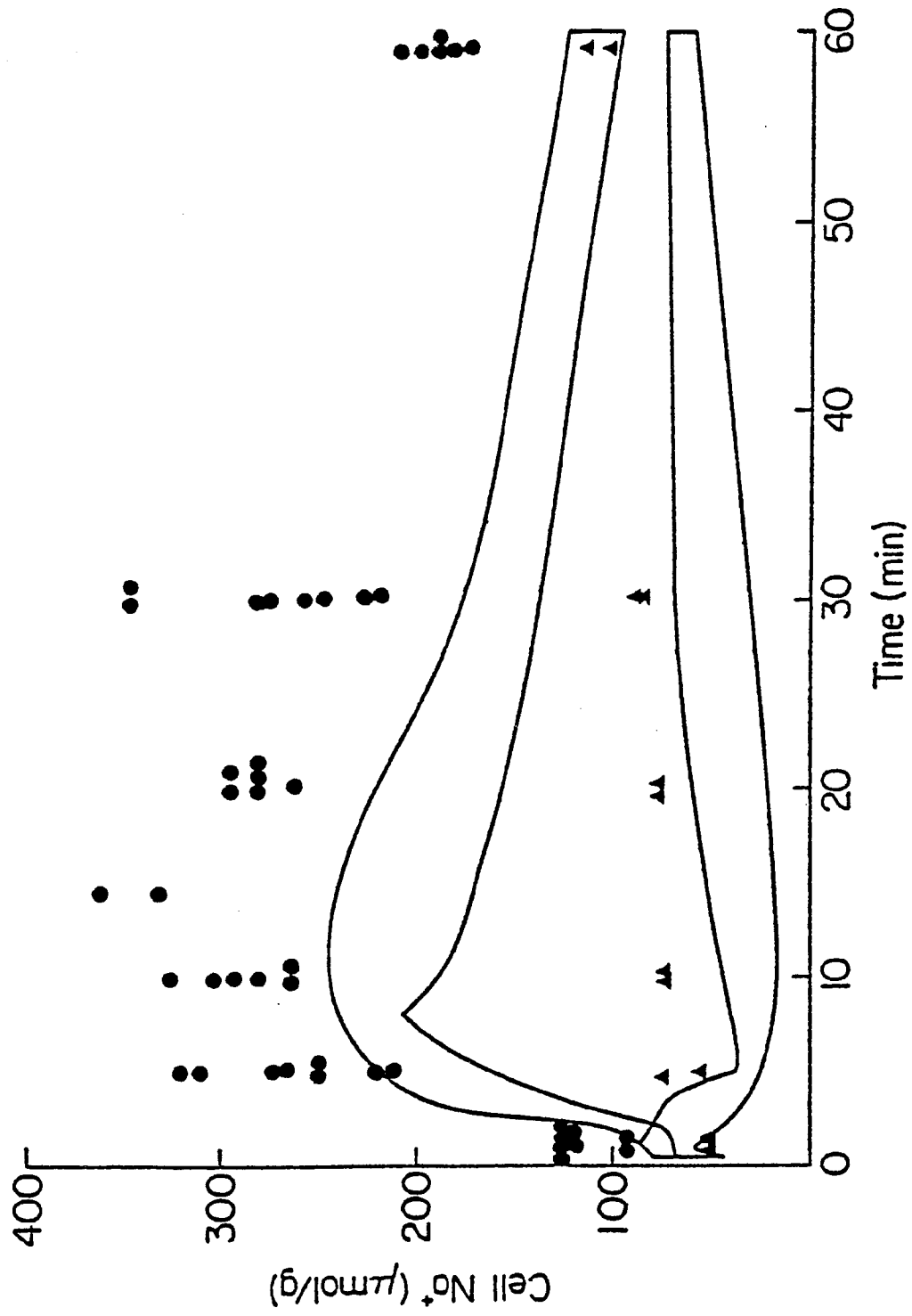


Figure 20. Effect of ouabain on cell Na^+. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) containing ouabain to produce the final suspension concentration of 0.1 mM. Cell Na^+ content in the presence of ouabain is compared to the 95% confidence bounds (solid lines) derived for control cells (without ouabain, figure 12).



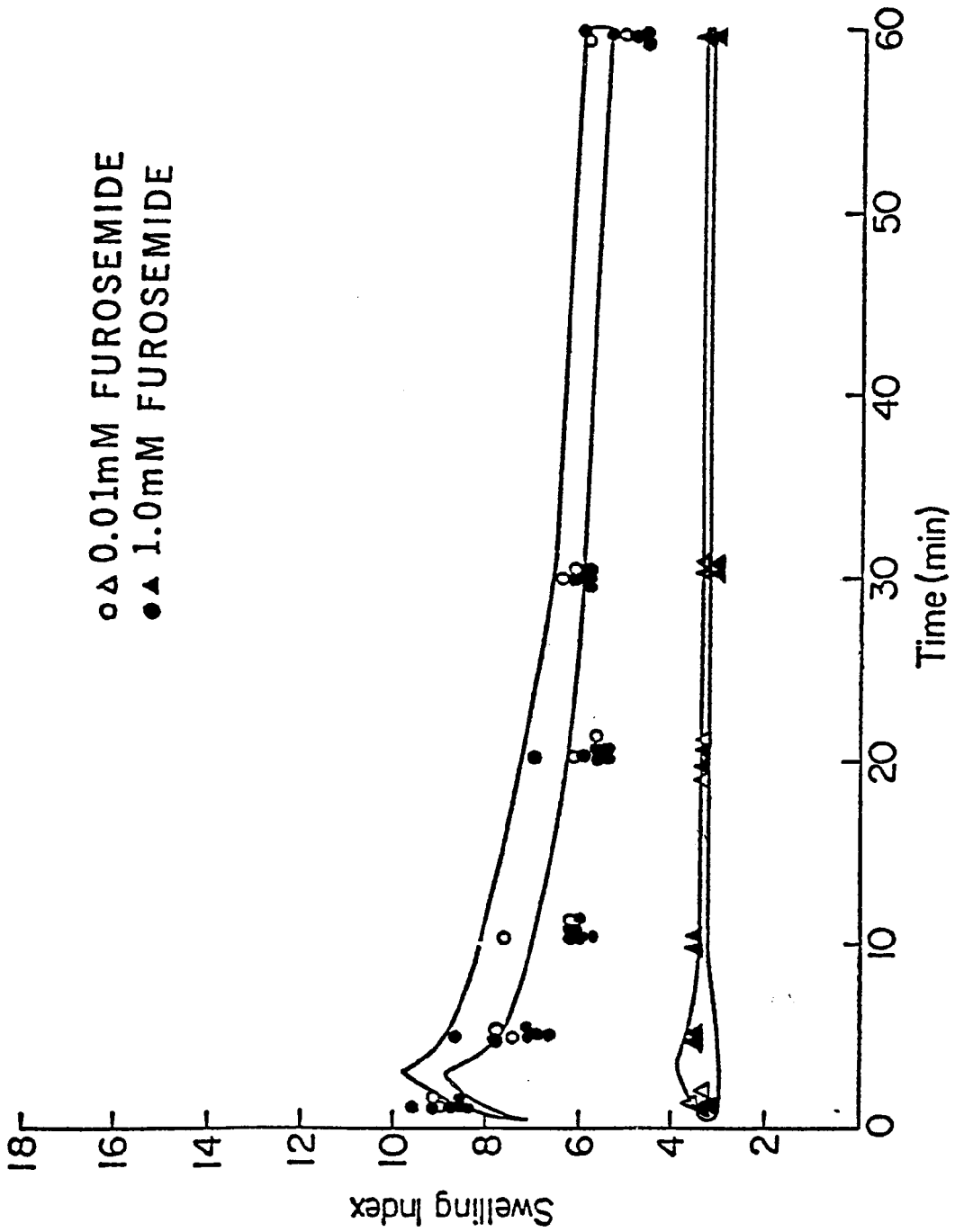
Effect of Ouabain on Cell Sodium in Isotonic (▲) and Hypotonic (●) Media

Since both furosemide and ethacrynic acid affect ion movements in transporting cells (Adragna et al. 1980, Seely and Dirks 1977, Ferguson and Twite 1974), their effect on the volume regulatory decrease in Ehrlich cells was tested. Furosemide is a weak acid and its actions are not limited to a single ion. When either 0.01 or 1.0 mM concentrations of furosemide were added to the sidearms prior to incubation, the swelling indices of the control cell was unaffected. However, the rate of volume regulation for the stressed cells was more rapid (figure 21). A large Na^+ loss and a slight K^+ increase occurred concurrently with the decrease in swelling of these cells (figures 22 and 23). When 1.0 mM furosemide was added to stressed cells in Na^+ free (K^+ replaced) media, the volume regulatory decrease, the changes in Na^+ content, and the changes in K^+ content were similar to those seen in the Na^+ free (K^+ replaced) experiments without furosemide.

Hoffman and Kregenow (1966) observed that the ouabain insensitive component of Na^+ efflux was sensitive to ethacrynic acid, even though ethacrynic acid had non-specific effects on cell metabolism (Daniel et al. 1971, Epstein 1972). When 1.0 mM ethacrynic acid was added to the sidearms of Na-KRP suspended cells, the control cells shrank and the volume regulatory decrease was lost after thirty minutes (figure 24). An increase in Na^+ and a decrease in K^+ occurred in both control and stressed cells when compared to the ninety-five percent confidence intervals of cells incubated without the inhibitor present (figures 25 and 26).

The pharmacological effects of colchicine probably result from its interaction with the microtubular protein, tubulin (Owellan et al. 1974). However, colchicine had no effect on either volume or ions in these studies.

Figure 21. Effect of furosemide on cell swelling index. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) containing furosemide to produce the final suspension concentrations indicated. Cell swelling indices in the presence of furosemide is compared to the 95% confidence bounds (solid lines) derived for control cells (without furosemide, figure 10).



Effect of Furosemide on Cell Water in Isotonic (▲,Δ) and Hypotonic (●,o) Media

Figure 22. Effect of furosemide on cell K^+. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) containing furosemide to produce the final suspension concentrations indicated. Cell K^+ content in the presence of furosemide is compared to the 95% confidence bounds (solid lines) derived for control cells (without furosemide, figure 11).

Effect of Furosemide on Cell Potassium in Isotonic (▲,△) and Hypotonic (●,○) Media

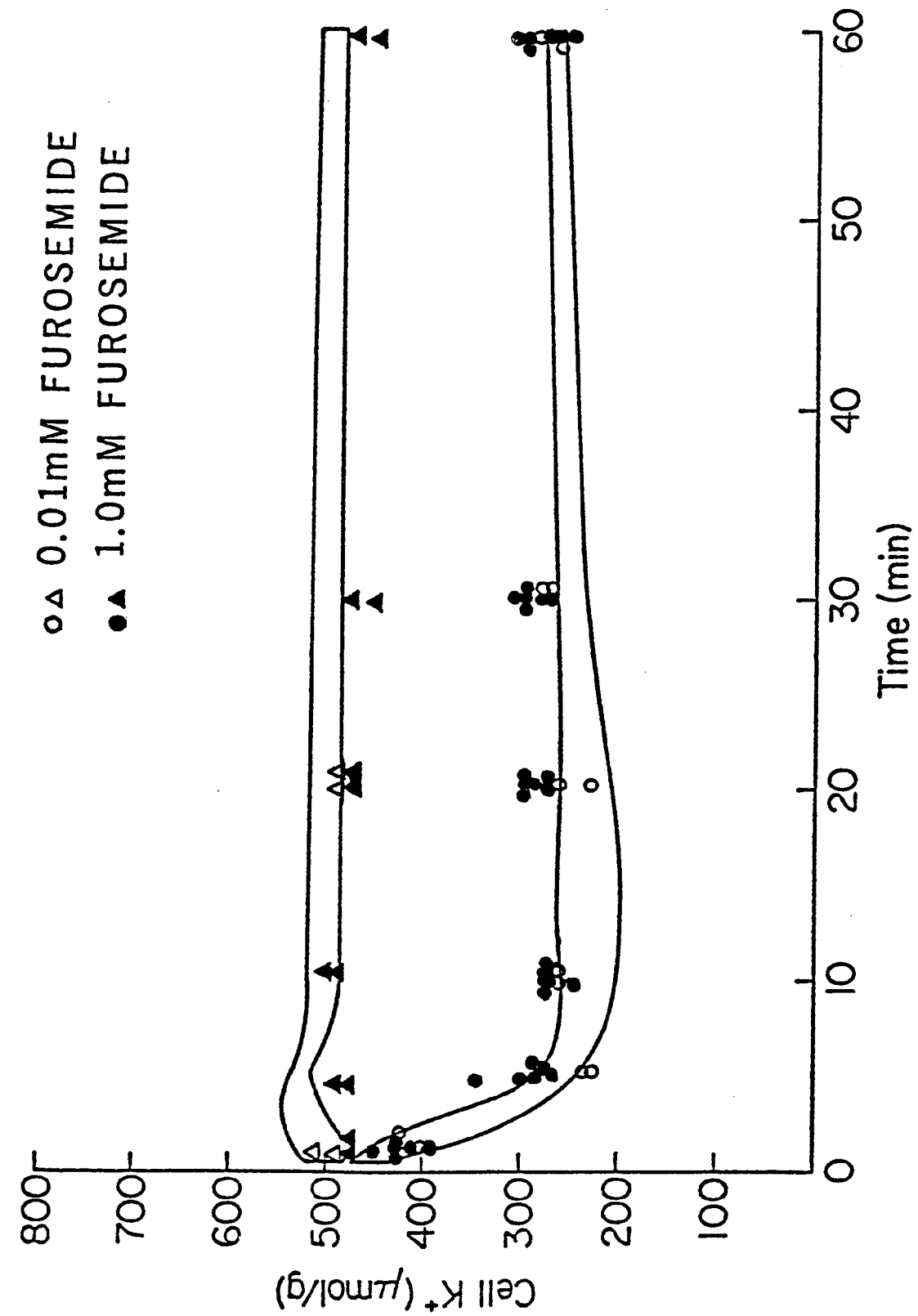
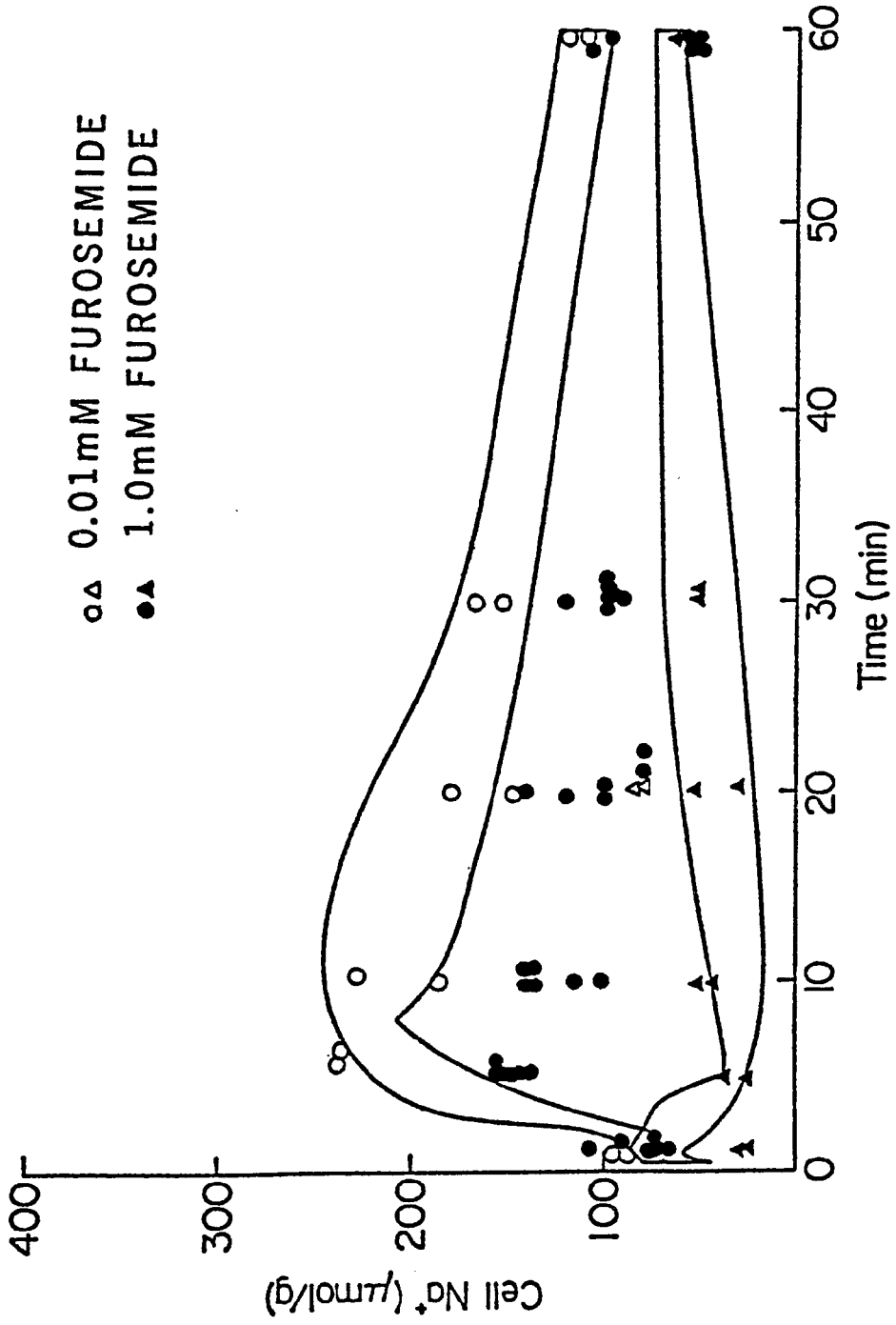


Figure 23. Effect of furosemide on cell Na^+. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) containing furosemide to produce the final suspension concentrations indicated. Cell Na^+ content in the presence of furosemide is compared to the 95% confidence bounds (solid lines) derived for control cells (without furosemide, figure 12).



Effect of Furosemide on Cell Sodium in Isotonic (▲,Δ) and Hypotonic (●,o) Media

Figure 24. Effect of ethacrynic acid on cell swelling index. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) containing ethacrynic acid to produce the final suspension concentration of 1.0 mM. Cell swelling indices in the presence of ethacrynic acid are compared to the 95% confidence bounds (solid lines) derived for control cells (without ethacrynic acid, figure 10).

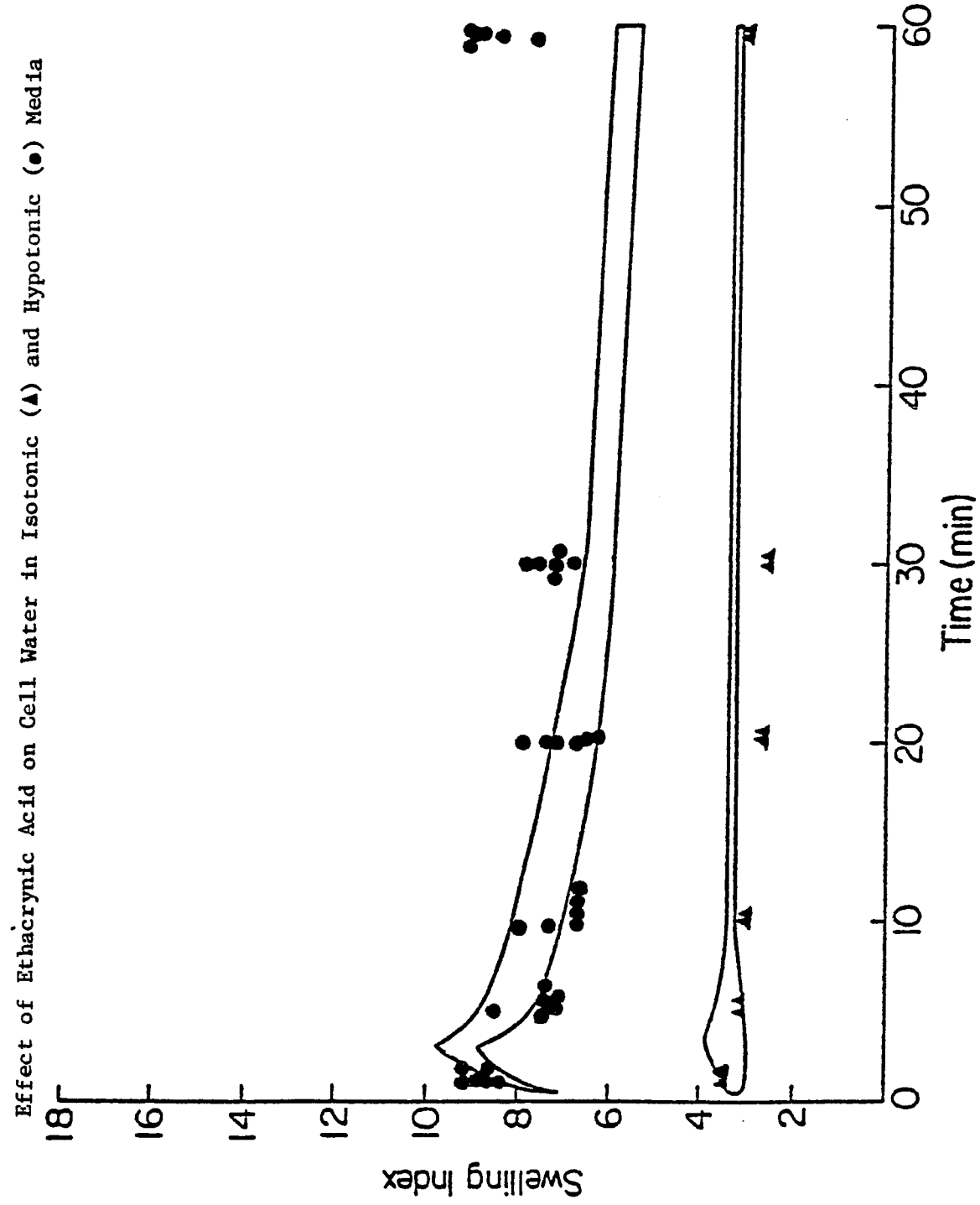

Effect of Ethacrynic Acid on Cell Water in Isotonic (▲) and Hypotonic (●) Media
Swelling Index
Time (min)
0
10
20
30
40
50
60
2
4
6
8
10
12
14
16
18

Figure 25. Effect of ethacrynic acid on cell K^+. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) containing ethacrynic acid to produce the final suspension concentration of 1.0 mM. Cell K^+ content in the presence of ethacrynic acid is compared to the 95% confidence bounds (solid lines) derived for control cells (without ethacrynic acid, figure 11).

Effect of Ethacrynic Acid on Cell Potassium in Isotonic (▲) and Hypotonic (●) Media

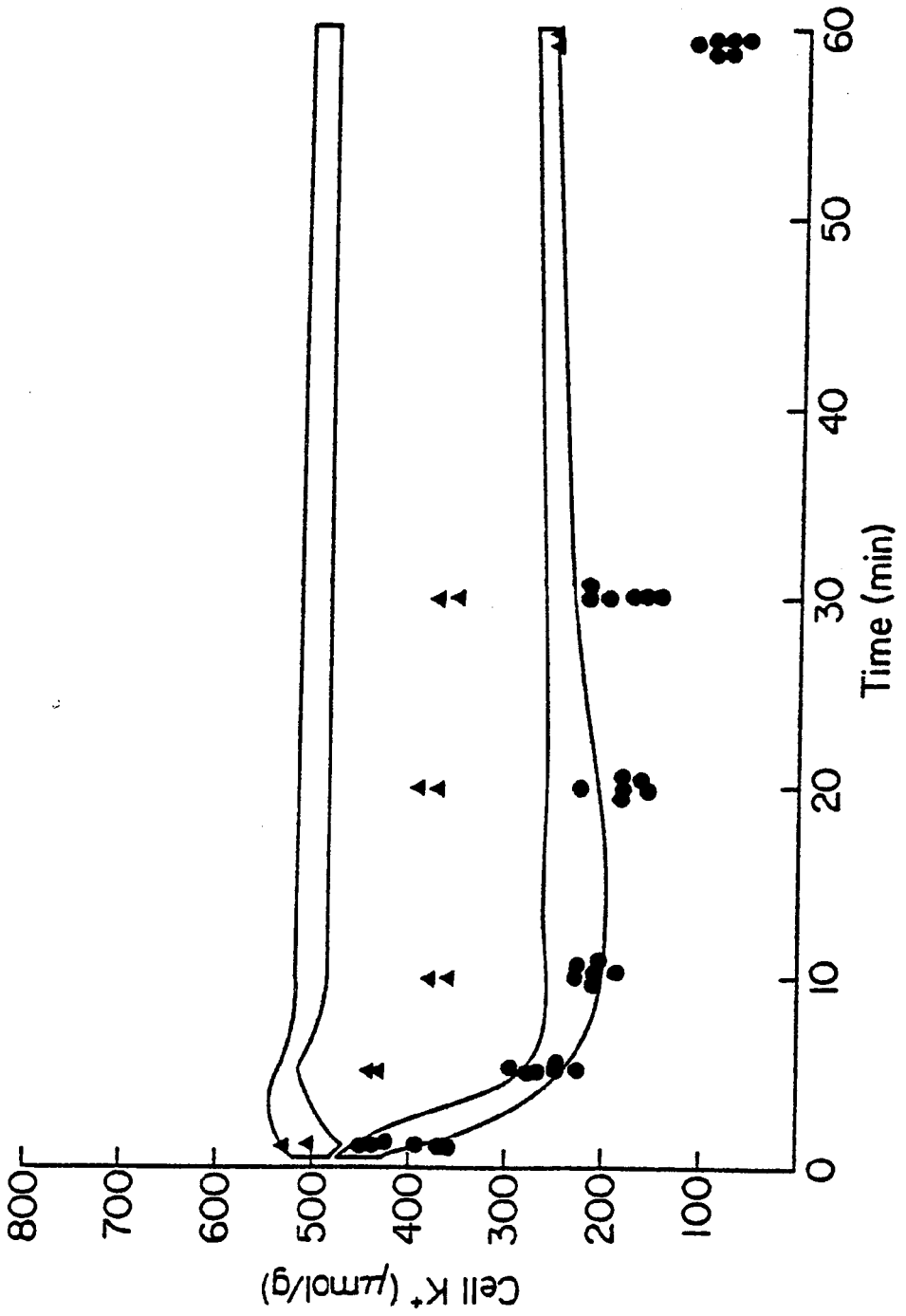
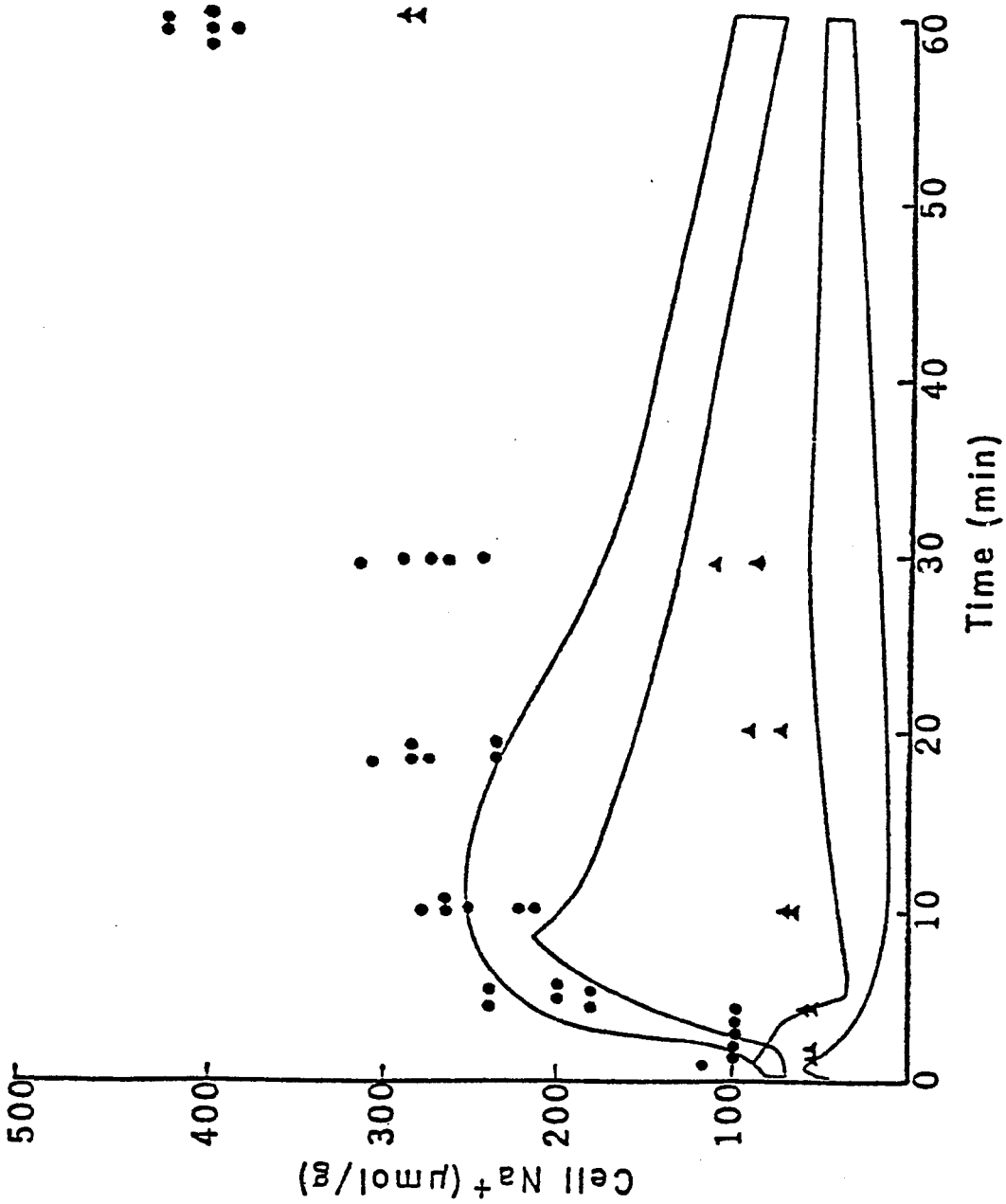


Figure 26. Effect of ethacrynic acid on cell Na^+. At time zero cell suspensions were diluted with either isotonic Na-KRP (triangles) or water (circles) containing ethacrynic acid to produce the final suspension concentration of 1.0 mM. Cell Na^+ content in the presence of ethacrynic acid is compared to the 95% confidence bounds (solid lines) derived for control cells (without ethacrynic acid, figure 12).

Effect of Ethacrynic Acid on Cell Sodium in Isotonic (▲) and Hypotonic (●) Media



DISCUSSION

RAPIDITY AND EXTENT OF INITIAL VOLUME CHANGES

The membrane of Ehrlich cells is highly water permeable, as evidenced by the rapidity of the initial volume increase when the cells are hypotonically stressed. As is illustrated in figure 7, the cells achieved ninety percent of their peak volume within less than sixty seconds after being placed in the 98 mosm/kg medium. Water movement across the cell membrane is governed by osmotic forces which are dependent on intra- and extracellular solute concentrations. Cells stressed by immersion in 98 mosm/kg medium (one third of the osmolality of the control medium, 294 mosm) would be expected to increase their volume exactly threefold if they behaved as perfect osmometers. However, the quantity of water that actually entered the cells, a 2.7-fold volume increase, was slightly less than the amount which would have been expected to pass through a semipermeable membrane as a result of osmosis. Several factors could account for this difference. Foremost, of course, a cell cannot be a perfect osmometer, because the cell membrane would have to be semipermeable and because intra- and extracellular fluids would have to behave as ideal solutions. In addition, Hoffmann and her colleagues (1979) suggest that the osmotic coefficient of proteins decreases and protein charges change with decreasing protein concentrations when Ehrlich cells swell. In contrast, they considered rapid leakage of ions, loss of solute, hydrostatic pressure created by the elastic cell membrane, and binding of water by intracellular large molecules to be of little significance. Other investigators have also observed less than perfect osmotic

behavior in other cell suspensions (Lucké and McCutcheon 1932, Kregenow 1971, Rorive and Gilles 1979).

VOLUME REGULATORY DECREASE

The second phase of volume change (the volume regulatory decrease) began approximately three minutes after cell dilution (figure 10). The rate of decline was rapid initially but subsequently slowed. The average swelling index for control cells at one minute was 3.12 while that for hypotonically stressed cells was 8.28, an increase to 265 percent of the original volume. By thirty minutes the stressed cells had eliminated only thirty-eight percent of the volume increase, and volume regulation was not complete even after sixty minutes. However, the magnitude and rate of the volume regulatory decrease seen in the present studies is comparable to that observed by Hendil and Hoffman (1974) in their studies with Ehrlich cells.

Although volume control in erythrocytes has been extensively studied, the red blood cell studies cannot be quantitatively correlated with the present studies. Red blood cells exposed to nonhemolytic hypotonic medium also swell and exhibit volume regulation, although like Ehrlich cells, they apparently are unable to restore their volume completely back to baseline levels. However, the rate and magnitude of the volume regulatory decrease in erythrocytes appears to differ from that in Ehrlich cells. The magnitude and the rate of decline observed by Cala (1977) in flounder red blood cells and in Amphiuma red blood cells (Cala 1980a) was less than that shown in figure 10. In contrast, Kregenow (1971) found a greater magnitude and rate of decline in duck red cells over a similar time period. During the hypotonic stress, K^+ content fell approximately 237 μmol/g and Na^+ content increased by approximately 59 μmol/g, producing a total intracellular cation decrease of approximately 178 μmol/g (table V). During the same time period, the intracellular volume decreased by 3.6 ml/g. If the

intracellular osmolality remained the same as that of the medium (98 mos/kg) during the volume regulatory decrease, the total cell osmoles must have decreased by 353 μmol/g (3.6 ml/g X 98 μmol/ml). Therefore, the net cation exit (178 μmol/g) and accompanying monovalent anions (178 μmol/g) can quantitatively explain the volume adjustment seen.

ION SHIFTS

During the initial or volume expansion phase of hypotonic stress K^+ content began to decrease almost immediately, falling from approximately 500 μmol/g at time zero to 400 μmol/g at two minutes. Cell volume began to decrease after three minutes, but K^+ continued to decrease further to a nadir of approximately 233 μmol/g at twenty minutes. It then increased very slightly during the remaining phase of volume regulation, and was approximately 270 μmol/g at sixty minutes (figure 27).

The loss of KCl from the swollen cell during the volume regulatory decrease could be either active or passive. The K^+ chemical gradient is large enough to support passive movement. This gradient is outwardly directed both with the initial dilution (60 meq/l intracellular to 5 meq/l extracellular) and during the volume regulatory phase (30 meq/l intracellular to 5 meq/l extracellular) (table V). As discussed previously, K^+ movement during volume regulation in other high K^+ cells is also thought to be passive. Other investigators have found that the passive loss of KCl during volume regulation was accompanied by increased K^+ permeability (Kregenow (1971) in duck red cells, Roti Roti and Rothstein (1973) in mouse leukemic cells, Hoffman and Hendil (1976) in Ehrlich cells, and Bui and Wiley (1981) in human lymphocytes). In the oocyte, hyperpolarization accompanies the volume regulatory decrease in hypotonic media -- more evidence to suggest the loss of intracellular K^+ is the consequence of changes in selective permeability (Sigler and Janacek 1971).

Table V. Comparison of Na^+ and K^+ concentrations and contents prior to and during volume regulation. Composite of morphologic changes, volume changes, and ion changes at the times indicated.

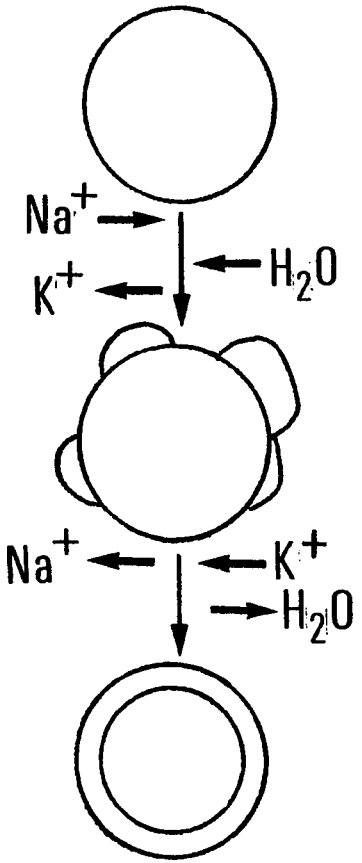


TABLE V

TIME	VOLUME	Na^+		K^+	
(min)	(ml/g)	Content (μeg/g)	Conc (mM)	Content (μeg/g)	Conc (mM)
0	3.1	54	17	501	155
1	8.3	75	9	453	55
10	7.6	216	28	238	31
60	5.7	111	19	271	48

Additionally, Dellasega and Grantham (1973) have observed that ouabain inhibits active K^+ transport in collecting tubules without affecting volume regulation, making it unlikely that active K^+ extrusion is involved in volume regulation.

If the volume regulatory decrease were dependent on passive K^+ efflux, then it might be expected to occur more rapidly with decreased extracellular K^+ concentrations. This effect has been seen by Bui and Wiley (1981) in human lymphocytes incubated in K^+ free medium. In the present studies, however, low concentrations or absent external K^+ did not affect volume regulation in hypotonically stressed Ehrlich cells until very late in the experiment. The swelling indices fell within the ninety-five percent confidence limits calculated for stressed cells incubated in Na-KRP except at sixty minutes. At that point, the indices were slightly below the confidence bounds. These findings were contrary to those of Rosenberg and his colleagues (1972) who found that Ehrlich cells were unable to volume regulate in K^+ free media. This discrepancy could possibly be explained by their use of cells which were K^+ depleted. If the initiation of the volume regulatory decrease is dependent upon passive K^+ efflux, it would be expected to be inhibited under such experimental conditions.

In K^+ free medium the stressed cells gained more Na^+ and lost more K^+ then they did when incubated in the presence of K^+. However, because the Na^+ gains above the ninety-five percent confidence limits approximately equaled the K^+ losses, the time course of the ion changes was the same whether K^+ was present or not.

Part of the ion imbalance seen in K^+ free media in the present studies might have been caused by interference with the pump. Hoffman (1977) showed that the Na^+/K^+ pump was operated in reverse in K^+ free (Na^+ replaced) media. The physical changes involved with cell swelling might also have affected the

pump or caused changes in the passive leak permeability for Na^+ which, along with the increased gradient, could also account for part of the ion movement.

THE ROLE OF NA^+ IN VOLUME REGULATORY DECREASE

In response to hypotonic stress cellular Na^+ content was initially increased, and the increase continued into the initial period of volume adjustment. However, during later volume regulation, Na^+ was extruded. Immediately after exposure to the hypotonic medium, intracellular Na^+ content increased from approximately 50 μmol/g to slightly over 200 μmol/g by ten minutes and then decreased toward baseline (table V). If Na^+ were not extruded during the period when volume adjustment would normally occur, volume regulation was impaired or lost. Ames and his colleagues (1965) observed similar ion and water movements in hypotonically stressed neural tissue. Even though Kregenow (1981) did not find any changes in Na^+ movement in duck red cells during hypotonic stress, he did observe a small Na^+ uptake in hypotonically stressed flounder and amphiuma red cells. He postulated that this increase in Na^+ would be immediately removed by a stimulated Na^+/K^+ pump. However, in the present studies, ion movement in cells incubated with ouabain followed the same time course as those incubated without ouabain, implying that Na^+ extrusion during volume regulation is not related to the Na^+/K^+ pump. Like the K^+ outflux, this previously unreported Na^+ influx is probably passive because the intracellular Na^+ concentration was less than the extracellular Na^+ concentration throughout the experimental procedure. The peak intracellular Na^+ concentration at ten minutes was approximately 29 meq/l when the external concentration was approximately 45 meq/l. These results imply that Na^+ permeability is increased with swelling or with initiation of volume regulation and K^+ exit.

Whittembury and Grantham (1976) postulated an essential role for Na^+ in volume regulation. They observed that hypothermia interfered with volume regulation and caused an increase in Na^+ content in hypotonically stressed renal cells. However, when these cells were subsequently warmed to 37° C, they lost Na^+ and volume regulated. The authors postulated that this inability to volume regulate in the cold reflected an inability to extrude intracellular cation and suggested that Na^+ was essential for net solute extrusion during the volume regulatory decrease. They suggested that hypothermic slowing of passive and pump fluxes in renal tissue and blocking of volume regulatory decrease indicates that both energy and increased K^+ permeability are necessary for volume regulation to occur. Hoffmann (1978) postulated that swelling in hypotonically stressed Ehrlich cells and the net loss of K^+, Cl^-, and water resulted from an increase in K^+ and Cl^- permeability, a decrease in Na^+ permeability, and a decrease in activity of the Na^+/K^+ pump. The rise in intracellular Na^+ seen in the present studies could not be explained by a decrease in Na^+ permeability.

Low concentrations of amiloride have been observed to interfere with Na^+ movement through specific conductive channels in certain nonepithelial cells. Additionally, high concentrations of amiloride block Na^+/H^+ electroneutral cotransport in certain leaky epithelia (Benos 1982). A role for this cotransport system in both intracellular volume regulation and intracellular pH regulation has been postulated. If Na^+ movement during volume regulation were through either system, amiloride might be expected to alter volume adjustment. Amiloride was studied at several different concentrations because the effects in different cells and species are variable. None of the three concentrations used (0.01, 0.1, and 1.0 mM) had any effect on the time course or magnitude of either the increased Na^+ content or the volume regulatory decrease in Ehrlich cells (figures 15 to 17). The higher concentrations resulted in

a slight increase in intracellular Na^+ and decrease in K^+ in stressed cells (figures 16 and 17) similar to that seen with ouabain and with K^+ free media. Similarly, Macknight and Leaf (1977) could find no evidence of inhibition of the volume regulating mechanism in renal cortex slices in the presence of amiloride. The results suggest that specific, amiloride-sensitive Na^+ channels are not involved in the Na^+ influx and argue against a Na^+/H^+ exchange mechanism. It is also possible that Na^+ competition interfered with amiloride inhibition, but since decreased levels of extracellular Na^+ inhibited volume regulation, it was not possible to test this alternate hypothesis by reducing extracellular Na^+.

With hypotonic stress, intracellular K^+ decreases, and Na^+ increases, which should stimulate the Na^+/K^+ pump. During the second phase of ion changes, Na^+ content was reduced and K^+ content was increased, suggesting active Na^+/K^+ ATPase pumping since both cations are moving against their electrochemical gradients. Ouabain, however, had no effect on volume regulation during either the initial (apparently passive) or final (possibly active) stage of ion adjustment (figure 18). However, ouabain did increase the gain in Na^+ above the ninety-five percent confidence bounds and thus obligated a greater loss of K^+ (figures 19 and 20), resulting in a normal volume decrease time course. Intracellular Na^+ content rose, peaked at approximately ten minutes and fell toward baseline, while K^+ content fell for approximately thirty minutes before beginning a slow rise. Clearly the pump was either not completely inhibited or was not necessary for Na^+ reduction during volume regulation.

The ion changes in the ouabain inhibited, hypotonically stressed cells were almost identical to those seen in hypotonically stressed cells in K^+ free media. These findings are in agreement with those of Kregenow (1974) with duck red cells. He concluded that either ouabain or K^+ free media block the Na^+/K^+

pump, and that passive ion leaks would have to account for the ion changes in the presence of such inhibition. Ouabain treatment without permeability changes should lead to cellular swelling, since Na^+ would leak into the cell, water would follow, and K^+ would be diluted in addition to leaking out. Therefore, the absence of volume changes would have to be the result of leak permeability changes after pump blockade (MacRobbbie and Ussing 1961). These findings are in opposition to those of Rosenberg and his colleagues who observed that oxygenated Ehrlich cells were unable to volume regulate in the presence of ouabain, but their results may be related to their use of cells which were K^+ depleted. They found ion changes similar to those shown in figures 19 and 20 in hypotonically stressed Ehrlich cells, but in contrast observed no volume regulation. (They also did not observe volume regulation in hypotonically stressed Ehrlich cells in K^+ free media.)

In the present studies cells were pre-incubated in an aerated, 37° C buffer and allowed to come to a steady state before manipulation so that subsequent changes could be compared to steady state data. Different results have been obtained when the prior incubation conditions differed. Roti Roti and Rothstein (1973) observed that preincubation of Ehrlich cells in ouabain or in K^+ free media for several hours, caused a decrease of the intracellular ratio of K^+/Na^+ and did not abolish volume regulation but caused it to diminish. Whittembury and Grantham (1976) and Grantham and his colleagues (1977) observed diminished volume regulation and a primary loss of Na^+ rather than K^+ in hypotonically stressed kidney tubules that had been preincubated for long periods of time with ouabain. They postulated that volume regulation involved either a ouabain insensitive pump or extrusion of NaCl in response to a hydrostatic pressure generated by the swelling of the cells.

The effects of ouabain seen in some preparations appear most likely to be secondary changes due to preparative procedures, and possibly could be reversed by preliminary incubation in oxygenated medium (Macknight and Leaf 1977). The inhibition of volume regulatory mechanisms by preincubation with ouabain might be explained by changes in normal cation gradients or ion ratios, since the addition of ouabain after the preincubation period does not have any effect on volume regulation. If a specific intracellular K^+/Na^+ ratio is necessary for the initiation of volume adjustment and the ratio is already decreased, as it was in the experiments of Roti Roti and his colleagues (1973), volume regulation would not be as effective. As discussed previously, the ratio in the present studies went from approximately 6/1 at initial dilution to approximately 1/1 at the start of the second phase of ion changes (table V).

Interestingly, during cell division, blebs are formed and permeability changes similar to those seen with hypotonic stress take place, and ouabain causes almost immediate cessation of cell division (Mayhew and Levinson 1968). Since ouabain does not penetrate the cell membrane significantly (Mayhew and Levinson 1968), it must have a membrane effect. Possibly a specific intracellular K^+ or Na^+ concentration or Na^+/K^+ ratio are required for normal cell division.

In the present studies volume regulation did not occur in Na^+ free medium. There is an inhibition of volume regulation whether Li^+, choline or K^+ replaces the extracellular Na^+. When choline or Li^+ were the substituting ion, there was still net K^+ loss after swelling, but there was a net K^+ increase when K^+ was the replacement ion. Control cells did not swell in choline or in Li^+ substituted media, but did swell in K^+ substituted isotonic media as would be expected from previous studies (Trump and Ginn 1968, Davson 1970, Potashner and Johnstone 1971). Many investigators have felt that the ability of elevated

external K^+ to block volume regulation in hypotonic media indicates that volume regulation is a consequence of passive K^+ efflux in association with an anion, presumably Cl^-. In the hypotonically stressed cells, K^+ replacement of Na^+ eliminated the favorable gradient for K^+ efflux. As discussed previously, in Ehrlich cells the intracellular K^+ concentration is approximately 60 meq/l initially and approximately 30 meq/l during the volume regulatory phase (table V) as compared to an extracellular concentration of approximately 5 meq/l. With K^+ replacement the extracellular concentration is approximately 50 meq/l. This observation lends support to the theory of passive movement of K^+ out of the cell during volume regulation and is compatible with the literature.

Control cells probably do not swell in choline and Li^+ substituted media because the cell membrane is impermeable to these ions. Loss of volume regulation by hypotonically stressed cells when Li^+ or choline replace Na^+ in the medium could be explained in two ways. The volume regulatory mechanism could require Na^+ exit after initial volume adjustment (at ten minutes, figure 12), but a nonspecific increase in membrane permeability could allow the normal intracellular Na^+ to exit immediately so that this cellular Na^+ is unavailable. Alternatively, a nonspecific increase in membrane permeability could allow Li^+ or choline to equilibrate across the membrane resulting in total cation increase, because Li^+ or choline entry was greater than K^+ plus Na^+ loss. An absolute requirement for Na^+ cannot be ruled out; however, the increase in K^+ exit beyond the ninety-five percent confidence limits, indicates that a rapid Li^+ or choline exchange for K^+, as seen in figure 14, must occur. A rapid influx of these cations into swollen cells could result from abnormally high non specific cation permeability due to the cell swelling. This influx could produce a net solute increase which would cause further swelling and loss of volume regulation

and is evidence that the increased Na^+ permeability seen with volume regulation may be a nonspecific effect of swelling.

Kregenow (1973) observed that duck erythrocytes swollen by suspension in isotonic, low Na^+ (K^+ substituted) media did not volume regulate until the medium Na^+ was replaced. He attributed this effect to the loss of the K^+ gradient, but such loss would not explain why choline or Li^+ substitution blocked volume regulation. A possible explanation for the effect of Na^+ free media is the reversal of the Na^+ electrochemical gradient.

Another transport system that has been implicated in volume regulation is the simultaneous electroneutral cotransport of Na^+, K^+, and two Cl^-, the $Na^+/K^+/2Cl^-$ cotransport described by Geck and Heinz (1980). This system is postulated to counteract the shrinking effect of the Na^+/K^+ pump by introducing osmotically active solute into the cell and thereby causing swelling. It is not obvious that this system could be involved in the volume regulatory decrease since the observed ion movements are not a cotransport of K^+ and Na^+ but rather countertransport. Nevertheless, the effect of furosemide, a specific inhibitor of the $Na^+/K^+/2Cl^-$ cotransport, was examined. In the presence of 1.0 mM furosemide, ion changes were diminished but volume regulation was faster. The increased rate of volume regulation appeared to be related to a quantitatively smaller biphasic response of Na^+ and a greater reduction of total cations earlier in the volume adjustment phase. In the presence of furosemide, the cells would have been expected to have an even greater increase in Na^+ than was seen. Both Na^+ and K^+ changes followed their normal time courses but the magnitude of the increase (Na^+) and decrease (K^+) was not as great. Macknight and Leaf (1977) also could find no evidence of inhibition of the volume regulatory mechanism in renal cortex slices in the presence of furosemide. Smith and Robinson (1981) have observed in cells with normal concentrations of

intracellular ions that furosemide inhibits Na^+/Na^+ and K^+/K^+ exchange but has no effect upon net ion movements.

Since K^+ movement out of the cell during volume regulation is greater than the Na^+ movement in, anion movement or movement of another cation must occur to preserve electroneutrality. Grinstein and his colleagues (1982) observed a volume induced increase of anion permeability in human lymphocytes. Additionally, Davis and Finn (1981) observed that volume regulation by frog urinary bladder, which had become swollen in response to hypotonic serosal bathing media, was inhibited by replacement of Cl^- with SO_4^{--}, by methylsulfate, by amiloride, or by ouabain. Since in the present studies, anion replacement in the extracellular buffer had no effect on volume regulatory decreases in Ehrlich cells, it was not possible to determine whether Cl^- was required for the volume regulatory decrease. High intracellular Cl^- has also been observed by other investigators during anion replacement studies in Ehrlich cells. Hempling (1958) observed that as much as forty percent of the original Cl^- remained in the cell when the cells were bathed in Cl^- free (sulfate replaced) media and as much as eighty percent of the Cl^- remained in the cell in NO_3^- replaced media.

Several hormones, including norepinephrine, are thought to exert their effects on electrolyte transport through the intermediacy of cAMP (Sutherland and Robinson 1966, Kregenow 1973, Kregenow et al. 1976). For example, Riddick and his colleagues (1971) and Kregenow and his coworkers (1976) observed that K^+ transport activated by norepinephrine in the duck erythrocyte appeared to be dependent upon elevated intracellular cAMP. Palfrey and his colleagues (1980) postulated that ion movement by $Na^+/K^+/2Cl^-$ cotransport is stimulated by cAMP. This would be expected to further increase cell swelling in hypotonically stressed cells. In contrast, increased intracellular cAMP has been

implicated in bleb resorption in hamster ovary cells. Porter and his colleagues (1974) observed that increased intracellular cAMP diminished blebs on hamster ovary cells, and that dibutyryl cAMP changed these cells from compact, randomly growing, blebbed cells to stretched, smooth, bipolar cells without blebs but with increased microvilli. Therefore, the responsiveness of transport processes in volume regulation to the cellular concentration of cAMP seemed a reasonable subject for investigation. However, dibutyryl cAMP had no effect on the ion or volume changes or on bleb formation in the hypotonically stressed Ehrlich cell.

MORPHOLOGIC EVENTS IN CELL SWELLING AND VOLUME REGULATORY DECREASE

When hypotonically stressed, Ehrlich cells swell and blebs form over most of their cell surface (figure 3C).

Bleb Formation

Morphometric studies demonstrated that bleb formation parallels volume and ion changes, even though the tumor cells were unable to completely volume regulate. Bleb areas increased the cell area enough to account for the increased volume. Measuring the areas was thought to give an accurate measure of volume because the two are arithmetically related. Doubling the volume ($radius^3$) of a sphere increases its surface area ($radius^2$) by a factor of approximately 1.6. During the volume regulatory decrease, cell areas varied directly with cell volumes as discussed below.

By light microscopy the cytoplasm of the cell appeared to be physically separated from that of the bleb, but transmission electron microscopy disclosed no physical barrier to the movement of cell organelles even though they remained segregated from the cytoplasm of the blebs (figure 5B). As discussed below, microtrabeculae could account for this separation.

Microtubule Inhibition

The mechanochemical hypothesis of volume regulation (Kleinzeller 1965) postulates that Ca^{++} and ATP metabolism control some type of "contractile process" that can generate enough tension to offset cellular colloid osmotic pressure by the maintenance of a hydostatic pressure gradient. It is highly unlikely that a major pressure gradient could exist across the cell membrane unless rigidity was imparted by a strong intracellular skeleton. Whether cytosketelal elements found in Ehrlich cells are capable of withstanding any major pressure gradients is unknown. However, the cortical cytoplasm of Ehrlich cells does contain a fine felt-work of filaments which extend into the microvilli and microtubules run parallel to the plasma membrane along the bases of the microvilli. Even though filaments have been observed to extend into the microvilli of unstressed cells, whether filaments are present in blebs is uncertain. Price (1967) observed bleb formation in human epithelioma cells during mitosis and could find no evidence that filamentous structures were involved in the formation of blebs, nor could he find evidence of fibers or microtubules entering the bleb. However, he did observe amorphous granular material and polysomes within the bleb cytoplasm. Bhisey and Freed (1975) reported similar findings.

Porter and Tucker (1981) described microtrabeculae which appear in electron micrographs as wispy amorphous structures that vary with cell shape, and postulated that the amorphous granular material other investigators had dismissed were such structures but that sections for transmission microscopy were so thin that the lattice character of the microtrabeculae was lost. They proposed that as cells approach the shape of a sphere (the lowest energy state) there is a sequential dismantling of internal skeletal structures. Microtubules disassemble and the microfilaments and microtrabecular lattice deform, leaving

gaps which allow Brownian motion of cytoplasmic organelles. Porter and Tucker suggested that this gap formation with increased Brownian motion only occurred at 4° C, but as described above, it occurrs in hypotonically stressed Ehrlich cells at 37° C. Additionally, Trump and Ginn (1968) noted Brownian motion of particles within cells swollen after suspension in 37° C, isotonic, low Na^+ (K^+ substituted) medium. Even though Porter and Tucker (1981) have not observed Brownian motion at 37° C, they have described unique and reversible structural changes in the cell lattice as a result of cell suspension in hypotonic media with the intertrabeculae spaces enlarging as the volume increases. These gaps produced by increased volume could explain the increased Brownian motion of organelles observed in Ehrlich cells when they were suspended in hypotonic media. A similar change in the lattice could account for the Brownian motion seen by Lewis (1923) when cells were made anoxic, were bathed in acidic pH, or underwent mitosis. Under all three of these conditions blebs are known to form. Brownian motion ceases after the mitotic cell divides or if the pH or oxygen content of the media is adjusted, which would also cause blebs to be resorbed.

As a test of Kleinzeller's mechanochemical theory Dellasega and Grantham (1973) studied renal tubule cells in hypotonic media to determine whether the inherent stiffness of the basement membrane generated hydrostatic gradients and restricted cellular swelling. They found that volume regulation was identical in the presence or absence of the basement membrane. In contrast, Grantham and his colleagues (1981) observed that hypotonically stressed renal cells of the proximal straight tubule which had been pre-incubated in ouabain did swell and volume regulate, but that removal of the basement membrane abolished the volume regulation, suggesting that membrane elasticity was involved in the volume regulatory decrease in the presence of ouabain.

Even though Rand (1964) suggests that the red blood cell is incapable of supporting hydrostatic pressures of the magnitude necessary to support the mechanochemical theory, he does postulate that elastic properties are responsible for the resistance of red blood cells to hypotonic media and observed that hyptonically stressed red cells formed very rigid spheres which relaxed due to elastic forces after several hours Rand and Burton (1964). However, in light of the present studies, it appears more likely that the relaxation he observed was due to volume regulation.

Little effort was made in the present studies to ascertain whether microfilamentous structural changes were involved in bleb formation, although changes in membrane structure resulting from cell swelling may be necessary for volume regulation. Cytochalasin B (42 μM), which interferes with contractile microfilaments, was added to the suspension medium for nonstressed cells and caused the rapid formation of small blebs (relative to those formed in hypotonic medium) in less than a minute. These blebs were reversed when cytochalasin B was removed and their formation was inhibited at 0^{o} C. Henius and his colleagues (1979) have observed that cytochalasin B has no effects on the morphology of subcellular components or permeability of cells to ^{3}H inulin and trypan blue. Colchicine, which disrupts microtubular structure and causes changes in cell shape, was added to suspension medium and stressed cells were incubated for periods up to one hour. There was no effect on bleb formation, ion changes, or the volume regulatory decrease, even though De Brabander and Borgers (1975) found complete disappearance of cytoplasmic microtubules by 120 minutes in $C_{3}H$ mouse cells incubated in similar concentrations of colchichine.

Although Penttila and Trump (1975a) observed no further ultrastructural changes in stressed, blebbed cells with up to four hours of incubation, in the present study two additional ultrastructural changes were

observed: a slight decrease in cell area (table IV) and a decrease in the number of intracellular lipid bodies. These changes were associated with a concomitant decrease in cell dry weight. The loss of lipid bodies could account for part of the decrease in weight and area. Other investigators also have observed variations in the number of lipid bodies in these cells during experimental manipulation. These lipid droplets are thought to be the main endogenous metabolic substrates in Ehrlich cells which contain little or no glycogen (Charlton and Wenner 1978, Nirenburg 1959). Selby and his colleagues (1956) found that the number of lipid bodies in Ehrlich cells increased with the number of days of intraperitoneal incubation. Spector (1975) observed that removal of large numbers of Ehrlich cells from the peritoneal cavity allowed the remaining cells to grow more rapidly, and in these favorable conditions, the number of lipid droplets was greatly decreased. In the present studies, lipid bodies may have been lost due to the similar beneficial effects of reduction in crowding and increase in available oxygen.

The rapidity of bleb formation which increases cell area by over fifty percent and is associated with changes in permeability -- a matter of less than a second for Ehrlich cells -- does not seem to allow time for the protein synthesis necessary for the production of new membrane which would presumably require synthesis of macromolecules. Therefore, these bleb formation and permeability changes appear to be the result of alterations in existing membrane.

With scanning electron microscopy it appeared that the blebs might be ballooned microvilli (figure 4B). The microvilli of Ehrlich cells have been estimated to increase their surface area by only thirty percent (Hempling 1958, Lassen et al. 1971), but the increase in cell surface area during hypotonic stress is greater than thirty percent indicating that the quantity of existing cell membrane would have been too small to cover all of the blebs. However, the

estimates of thirty percent were based on electron micrographs produced in the 1950's which depict cells with fewer microvilli than are seen with later, improved microscopic techniques. Knulton and his colleagues (1976) have observed that microvilli can increase the surface area of cells enough to allow at least a two to threefold increase in volume and suggest that swelling in cells with microvilli is associated with unfolding of these microvilli. Pasternak (1976) has observed that microvilli provide enough membrane to at least double the cell surface area and that the microvilli unfold to provide the extra surface area necessary to cover two daughter cells during cell division. Other investigators have estimated that microvilli increase the area of the cell surface severalfold. Blom and Helander (1977) have proposed that microvilli increase the surface area of gallbladder epithelial cells fivefold and Nellans and his coworkers (1974) have proposed an eightfold increase in the surface area of rabbit ileum due to the presence of microvilli. Therefore the microvilli contain more than enough cell membrane to form the blebs.

Trump and his colleagues (1979a, 1979b) have also postulated that blebs were formed from existing membrane. With transmission and scanning microscopy they found blebs similar to those described in this study following a variety of chemical and mechanical injuries. They postulated that blebs were bulbous enlargements of microvilli, that occassional elongated microvilli became protruding blebs, and that average microvilli formed short, thick blebs close to the cell surface. However, the studies described here indicate that low, thick blebs are a result of coalescence and partial retraction of the larger protruding blebs.

Immunocytochemical techniques were used to characterize the bleb membrane. Antibody to ATPase was used because Ehrlich cells are known to have Na^+/K^+ pumps in their plasma membranes and because Wood and his

colleagues (1977), in studies on tissues from the central nervous system, have demonstrated the feasibility of using immunocytochemical methods to localize Na^+/K^+ ATPase and its subunits. Additionally the tumor cells contain immunoglobulin proteins on their plasma membranes but not intracellularly (Mehrishi 1972, Garnis and Lala 1978) . In support of this Wallach and Kamat (1966), Wallach and his colleagues (1966), and Wallach and Vlahovic (1967) observed that (unidentified) antigenic sites on the plasma membrane were lacking on the smooth endoplasmic reticulum. In the present studies, both anti-ATPase and anti-IgG antibodies were used (figure 6). ATPase antibodies were found on the cell membrane and within the cell as expected because ATPase is found in the plasma membrane and in localized areas within the cell. IgG antibodies were scattered over the plasma membrane alone, which was also expected since these markers are assumed to be limited to the external membrane. Antibodies to both ATPase and IgG were observed on the blebs (figure 6), indicating the membranes were formed from pre-existing plasma membrane but not from smooth endoplasmic reticulum.

Blebs were always observed on cells suspended in hypotonic media, but they were also observed on approximately ten percent of the control cells suspended in isotonic media. The blebs on hypotonically stressed cells were numerous and often covered the entire external surface, while those on control cells were ususally smaller in size and fewer in number. No explanation for the blebs on the control cells is evident. Perhaps blebs on some cells are associated with mitosis, since heterogenous suspensions of cells in various stages of cell division were used in all studies, or perhaps some of the cells were injured during specimen preparation. A third possibility is that the cells formed blebs due to age--old erythrocytes are less resistant to hypotonic shock--or due to environmental factors. Chemnitz and Salmberg (1977) found that the number of

vacuoles, blebs, and annulate lamellae in Ehrlich cells increased rapidly after ten days of abdominal incubation. De Brabander and Borgers (1975) postulate that nuclear membrane blebs pinch off and fuse to make annulate lamellae, so an increase in annulate lamellae implies an increase in intracellular blebs in addition to the increase of plasma membrane blebs with age. Incubation time probably was not responsible for the bleb formation since the percentage of blebs on control cells did not increase with time. As stated, Penttilla and Trump (1975a) observed no ultrastructural changes in Ehrlich cells with four hours of incubation.

Bleb formation during hypotonic stress is similar to bleb formation when cells are anoxic, toxically inhibited, cooled to 0^{o} C, or injured chemically (Selby et al. 1956, Trump and Ginn 1968). Like the blebs seen on hypotonically stressed cells, the formation of blebs following metabolic inhibition or injury was reversed when the environment was returned to normal. Bleb formation in anoxic renal epithelial cells is reversible with oxygenation and blocked by polyethylene glycol, an oncotic agent (Frega et al. 1979, Kreisberg et al. 1980). This similarity indicates that bleb formation with hypotonic stress and metabolic inhibition is probably related to osmotic and volume changes.

Bleb Relation to Cell Volume Change

Cell perimeters, areas, and circularities were measured during morphometric studies. The accuracy of these measurements by light microscopy is limited by the resolution of light microscopes and the presence of numerous membrane infoldings and microvilli. In these studies, the cell images were focused at the maximal cell diameter which allowed precise measurements because cell outlines were sharp and clear. Repeated digital image intensification further increased the sharpness of the outline. Data obtained in these studies were merely estimates, since the true cell surface was covered

with microvilli and no effort was made to include these structures in the cell outline. However, since outline measurements of the same cell with and without blebs were used for comparisons, errors in measurements would be similar for each determination. Quantitative data was produced through mathematical manipulation and statistical analysis (Chalkley 1943, Weibel 1973) as described in RESULTS and statistical techniques aided in the determination of the correct dimensions of three dimensional structures.

Using these methods a mean control cell diameter of 15.6 μ and a cross sectional area of approximately 191.0 μ^2 were obtained. These measurements were similar to those obtained by other investigators with different techniques. Mehishi (1972) found a range of cell diameters of 12 to 20 μ, Smith and his colleagues (1972) a range of 8 to 24 μ, Smith and Vernon (1979) a range of 12 to 16 μ, and Hempling (1958) a mean value of 16 μ. Other investigators have measured cross sectional cell areas and obtained results similar to the mean of 191 μ^2. Hempling (1958) and Lassen and his colleagues (1971) found areas of 188 μ^2 while Maizels and his colleagues (1958a, 1958b) and Mehrishi (1972) both observed areas of 225 μ^2. Using gravimetric techniques in the present study, mean control cell volume was 1349 μ^3, and mean stressed cell volume was calculated as 2886 μ^3. Other investigators have arrived at calculated control volumes for nonstressed cells of 1610 μ^3 using occular micrometry (Laiho et al. 1971), and 1230 μ^3 using a hematocrit centrifuge (Mayhew et al. 1973).

In the present study the volume calculated from the radius derived from the measured control areas and perimeters assuming a sphere was approximately 2040 μ^3. Assuming an oblate ellipsoid the calculated volume was approximately 2435 μ^3 and assuming a prolate ellipsoid it was approximately 1712 μ^3. The value for a prolate ellipsoid most closely approximated the gravimetrically determined value. The 363 μ^3 difference between the two values could possibly

be accounted for by the contribution of osmotically inactive solute. After assuming that the cells had a spherical shape, Hempling (1958) and Maizels and his colleagues (1958a) directly measured cell diameters and calculated volumes of 2145 μ^3 and 2800 μ^3. The data gathered using morphometric techniques compared closely to that obtained using gravimetric measurements and determining the cell volumes by counting and directly weighing the cells allowed the calculations of cell shape to be independently checked.

Calculation of cell volumes from morphometric measurements of areas and perimeters while the volumes were changing allowed bleb formation to be correlated with volume changes. The mean one minute control cell area was 191 μ^2 and the gravimetrically measured volume was 1349 μ^3 while the stressed cell area increased approximately fifty percent above the control level to 298 μ^2 and the volume increased one hundred percent to a mean of approximately 2886 μ^3. The area to volume ratio of the one minute control cells was 0.1415 while that of the stressed cells was 0.1033. The area to volume ratio of the thirty minute stressed cells was also 0.1033 indicating both parameters were decreased at approximately the same rate. The thirty minute stressed cell area was 238 μ^2 with a volume of 2305 μ^3.

SUMMARY

The results of the present studies provide further information concerning the nature of the volume regulatory process in Ehrlich ascites tumor cells.

1. Hypotonic stress in Ehrlich ascites tumor cells was investigated.

2. These cells swelled rapidly due to high water permeability.

3. The following ion changes were observed to occur: (i) initial dilution of intracellular K^+ and Na^+ was followed by K^+ loss and Na^+ gain with K^+ loss exceeding Na^+ gain; (ii) the initial Na^+ gain followed by a loss was a new observation for volume regulation in these cells but was interpreted as most likely not significant, since the initial ionic permeability appeared to be general, allowing even choline to enter; and (iii) later, during the volume regulatory phase, a slight increase in K^+ content and a large decrease in Na^+ content, which was not ouabain sensitive, took place.

4. The volume regulatory decrease was not sensitive to amiloride, ouabain, dibutyryl cAMP, or colchicine; but may have been slightly accelerated by furosemide. It was inhibited by ethacrynic acid and by Na^+ replacement in the external medium which resulted in either a loss of the K^+ gradient of an increase in the intracellular ions which in turn nullified the K^+ exit effect.

5. Morphologic changes occurred concurrently with ion changes. Initial swelling was accompanied by the formation of plasma membrane blebs.

Bleb formation and reduction were interpreted to be directly related to the volume increase and the volume regulatory decrease . Blebs were formed from pre-existing membrane. Microvilli area was sufficient to account for bleb formation. The volume represented by the newly-formed blebs was found by morphometry to be equal to the volume gained during hypotonic swelling. As the volume of the cells decreased during volume regulation, the blebs were observed to coalesce into a halo around the cytoplasmic perimeter.

6. The results support the concept that changes in membrane permeability to K^+, Cl^-, and Na^+ underlie volume control in hypotonic medium.

BIBLIOGRAPHY

Adragna, N., M. Canessa, I. Bize, R. Garay and D.C. Tosteson. 1980. (Na + K) co-transport and cell volume in human red cells. Fed. Proc. 39:1842 (Abstr.).

Agnish, N.D. and S. Fedoroff. 1968. Tumor cell population of the Ehrlich ascites tumors. Canad. J. Genet. Cytol. 10:723-746.

Allen, R.D., G.B. David, and G. Nomarski. 1969. The Zeiss-Nomarski differential interference equipment for transmitted light microscopy. Z. Wiss. Mikrosk. 69(4):193-220.

Ambrose, E.J. 1966. Surface properties of tumor cells. In: Biology of Cancer. E.J. Ambrose and F.J.C. Roe, eds. D. Van Nostrand, London. 27-57.

Ames, A, III, J.B. Isom, and F.B. Nesbett. 1965. Effects of osmotic changes on water and electroltyes in nervous tissue. J. Physiol. (London) 177:246-262.

Beams, H.W. and R.G. Kessel. 1968. Properties of the Ehrlich ascites tumor cells as determined by electron microscopy, ultracentrifugation and hydrostatic pressure. Cancer Res. 28:1944-1951.

Belkin, M. and W.G. Hardy. 1961. Relation between water permeability and integrity of sulfhydryl groups in malignant and normal cells. J. Biophys. Biochem. Cytol. 9:733-745.

Belkin, M. and M.J. Shear. 1938. Chemical studies on tumor tissue. VI. Comparative effects of serum colloids and of salts on the swelling of mouse tumor cells in vitro. Amer. J. Cancer 32:62-75.

Benos, D. J. 1982. Amiloride: a molecular probe of sodium transport in tissues and cells. Amer. J. Physiol. 242:c131-145.

Bhisey, A.N. and J.J. Freed. 1975. Remnant motility of macrophages treated with cytochalasin B in the presence of colchicine. Exp. Cell Res. 95:376-384.

Bisson, M.A. and J. Gutknecht. 1975. Osmotic regulation in the marine algae, Codium decorticatum. I. Regulation of turgor pressure by control of ionic composition. J. Membrane Biol. 24:183-200.

Blom, H. and H.F. Helander. 1977. Quantitative electron microscopical studies on in vitro incubated rabbit gallbladder epithelium. J. Membrane Biol. 37:45-61.

Bonting, S.L. and L.L. Caravaggio. 1966. Studies on Na^+-K^+-activated adenosine triphosphatase. XVI. Its absence from the cation transport system of Ulva lactuca. Biochim. Biophys. Acta. 112:519-523.

Boss, J. 1955. Mitosis in cultures of Newt tissues IV. The cell surface in late anaphase and the movements of ribonucleoprotein. Exp. Cell Res. 8:181-187.

Buckhold B., R.B. Adams, and E.C. Gregg. 1965. Osmotic adaptation of mouse lymphoblasts. Biochim. Biophys. Acta. 102:600-608.

Bui, A.H. and J.S. Wiley. 1981. Cation fluxes and volume regulation by human lymphocytes. J. Cell Physiol. 108:47-54.

Burrows, M.T. and C.A. Neyman. 1917. Studies on the metabolism of cells in vitro. I. The toxicity of alpha amino acids for embryonic chicken cells. J. Exp. Med. 25:93-108.

Cala, P.M. 1977. Volume regulation by flounder red blood cells in anisotonic media. J. Gen. Physiol. 69:537-552.

Cala, P.M. 1980a. Volume regulation by amphiuma red blood cells: the membrane potential and it's implications regarding the nature of the ion-flux pathways. J. Gen. Physiol. 76:683-708.

Cala, P.M. 1980b. Volume regulation by amphiuma red blood cells: the nature of the ion flux pathways. Fed. Proc. 39(3):379 (Abstr.).

Canessa, M., I. Bize, N. Adragna, and D. Tosteson. 1982. Cotransport of lithium and potassium in human red cells. J. Gen. Physiol. 80:149-168.

Chalkley, H.W. 1943. Method for the quantitative morphologic analysis of tissues. J. Nat. Cancer Inst. 4:47-53.

Chan, P.C., V. Calabrese, and L.S. Theil. 1964. Species differences in the effect of sodium and potassium ions on the ATPase of erythrocyte membranes. Biochim. Biophys. Acta 79:424-426.

Charlton, R.R. and C.E. Wenner. 1978. Calcium-ion transport by intact Ehrlich ascites-tumor cells: Role of respiratory substrates, Pi, and temperature. Biochem. J. 170:537-544.

Chayes, F. 1965. Determination of relative volume by sectional analysis. Lab. Invest. 14:987-995.

Chemnitz, J. and K. Salmberg. 1977. Interrelationship between annulate lamellae and the cytoplasmic microtubule complex in tumor cells in vivo and in vitro. S. Krebsforsch 90:175-185.

Chiang, J., M. Kowada, A. Ames, 3rd, R.L. Wright and G. Majno. 1968. Cerebral ischemia. III. Vascular changes. Amer. J. Pathol. 52:455-476.

Costero, I. and C.M. Pomerat. 1951. Cultivation of neurons from the adult human cerebral and cerebellar cortex. Amer. J. Anat. 89:405-468.

Daniel, E.E., A.M. Kidwai, K. Robinson, D. Freeman, and S. Fair. 1971. The mechanisms by which ethacrynic acid affects ion content, ion fluxes, volume and energy supply in rat uterus. J. Pharmacol. Exp. Ther. 176(3):563-579.

Danowski, T.S. 1941. The transfer of potassium across the human blood cell membrane. J. Biol. Chem. 139:693-705.

Darrow, D.C. and H. Yannet. 1935. The changes in the distribution of body water accompanying increase and decrease in extracellular electrolyte. J. Clin. Invest. 14:266-275.

Davies, W.A. and T.P. Stossel. 1977. Peripheral hyaline blebs (podosomes) of macrophages. J. Cell Biol. 75:941-955.

Davis, C.W. and A.L. Finn. 1981. Regulation of cell volume of frog urinary bladder. In: Membrane Biophysics: Structure and Function in Epithelia. Alan R. Liss, Inc., New York. 25-36.

Davson, H. 1970. A Textbook of General Physiology, 4th Ed., The Williams and Wilkins Company, Baltimore.

Dean, R.B. 1941. Theories of electrolyte equilibrium in muscle. Biol. Symp. 3:331-348.

De Brabander, M. and M. Borgers. 1975. The formation of annulated lamellae induced by the disintegration of microtubules. J. Cell Sci. 19:331-340.

Delesse, M.A. 1847. Procede mecanique pour determiner la composition des roches. C.R. Acad. Sci. (Par.) 25:544-545.

Dellasega, M. and J.J. Grantham. 1973. Regulation of renal tubule cell volume in hypotonic media. Amer. J. Physiol. 224 (6):1288-1294.

DiBona, D.R. 1978. Direct visualization of epithelial morphology in the living amphibian urinary bladder. J. Membrane Biol. Suppl. 40:45-70.

DiBona, D.R., J.A. Schafer, T.A. Berglindh, and G. Sachs. 1981. The use of combined differential interference-contrast and fluorescence optics for analysis of epithelial transport. In: Epithelial Ion and Water Transport, A.D.C. Macknight and J.P. Leader (Eds.) Raven Press, New York.

Dick, D.A.T. 1971. Water movements in cells. In: Membranes and Ion Transport. E.E. Bittar, ed. Wiley Interscience, New York. 3:211-250.

Dörge, A., R. Rick, U. Katz, and K. Thurau. 1981. Determination of intracellular electrolyte concentrations in amphibian epithelia with the use of electron microprobe analysis. In: Water Transport across Epithelia. Alfred Benzon Symposium XV. H.H. Ussing, N. Bindslev, N.A. Lassen, and O. Sten-Knudsen, eds. Munksgaard, Copenhagen.

Donohoe, J.F., M.A. Venkatachalam, D.B. Bernard and N.G. Levinsky. 1978. Tubular leakage and obstruction after renal ischemia: structural-functional correlations. Kidney Int. 13:208-222.

Dornfeld, E.J. and A. Owczarzak. 1958. Surface responses in cultured fibroblasts elicited by ethylenediaminetetraacetic acid. J. Biophysic. Biochem. Cytol. 4:243-250.

Epstein, R.W. 1972. The effects of ethacrynic acid on active transport of sugars and ions and on other metabolic processes in rabbit kidney cortex. Biochim. Biophys. Acta. 274:128-139.

Ferguson, D.R. and B.R.Twite. 1974. Furosemide-pharmacology and cellular mode of action. Naunyn-Schmiedeberg's Arch. Pharmacol 281:295-300.

Follett, E.A.C. and R.D. Goldman. 1970. The occurrence of microvilli during spreading and growth of BHK21/C13 fibroblasts. Exp. Cell. Res. 59:124-136.

Frega, N.S., D.R. DiBona, and A. Leaf. 1979. The protection of renal function from ischemic injury in the rat. Pflugers Arch. 381:159-164.

Garnis, S. and P.K. Lala. 1978. Surface markers of small lymphocytes appearing in the mouse Ehrlich ascites tumour, host spleen and blood. Immunology 34(3):487-499.

Gary-Bobo, C.M. and A.K. Solomon. 1968. Properties of hemoglobin solutions in red cells. J. Gen. Physiol. 52:825-853.

Geck, P. and E. Heinz. 1980. Coupling of ion flows in cell suspension systems. Ann. N.Y. Acad. Sci. 341:57-66.

Geck, P., C. Pietrzyk, B.C. Burckhardt, B. Pfeiffer and E. Heinz. 1980. Electrically silent cotransport of Na^+, K^+, and Cl^- in Ehrlich cells. Biochim. Biophys. Acta 600:432-447.

Gilles, R. 1978. Intracellular free amino acid and cell volume regulation during osmotic stresses. In: Osmotic and Volume Regulation: Alfred Benzon Symposium XI. C.B. Jørgensen and E. Skadhauge, eds. Academic Press, New York. 470-491.

Goldman, R.D. E. Lazarides, R. Pollack, and K. Weber. 1975. The distribution of actin in non-muscle cells. The use of actin antibody in the localization of actin within the microfilament bundles of mouse 3T3 cells. Exp. Cell. Res. 90:333-344.

Gordon, M.S. 1965. Intracellular osmoregulation in skeletal muscle during salinity adaptation in two species of toads. Biol. Bull. 128:218-229.

Grantham, J., M. Linshaw, and L. Welling. 1981. Volume regulation in isotonic and hypotonic media in isolated rabbit renal proximal tubule. In: Epithelial Ion and Water Transport. A.D.C. Macknight and J.P. Leader, eds. Raven Press, New York. 339-347.

Grantham, J.J. 1977. Pathophysiology of hyposmolar conditions: a cellular perspective. In: Disturbances in Body Fluid Osmolality. T.E. Andreoli, J.J. Grantham, and F.C. Rector, Jr., eds. American Physiol. Soc., Bethesda, Md. 217-225.

Grantham, J.J., C.M. Lowe, M. Dellasaga, and B.R. Cole. 1977. Effect of hypotonic medium on K and Na content of proximal renal tubules. Amer. J. Physiol. 232:F42-F49.

Grinstein, S., C.A. Clarke, A. Dupre, and A. Rothstein. 1982. Volume-induced increase of anion permeability in human lymphocytes. J. Gen. Physiol. 80:801-823.

Gupta, J.D., V.J. Peterson, and J.D. Harley. 1974. Erythrocytic ouabain-sensitive and ouabain-insensitive adenosine triphosphatase in various mammalian species. Comp. Biochem. Physiol. 47A:1123-1126.

Haas, M., W.F. Schmidt, III, and T.J.McManus. 1982. Catecholamine-stimulated ion transport in duck red cells. Gradient effects in electrically neutral (Na + K + 2Cl) co-transport. J. Gen. Physiol. 80:125-147.

Hales, S. 1731-1733. Statical Essays: Containing Haemastaticks. W. Innys and R. Manby, London.

Harris, J.E. 1941. The influence of the metabolism of human erythrocytes on their potassium content. J. Biol. Chem. 141:579-595.

Heinz, A., J.W. Jackson, B.E. Richey, G. Sachs, and J.A. Schafer. 1981. Amino acid active transport and stimulation by substrates in the absence of a Na^+ electrochemical potential gradient. J. Membrane Biol. 62:149-160.

Heinz, E., P. Geck, C. Pietrzyk, and B. Pfeiffer. 1977. Electrogenic ion pump as an energy source for active amino acid transport in Ehrlich cells. In: Biochemistry of Membrane Transport. G. Semenza and E. Carafoli, eds. Springer-Verlag, New York. 234-259.

Hempling, H.G. 1958. Potassium and sodium movements in the Ehrlich mouse ascites tumor cell. J. Gen. Physiol. 41:565-583.

Hempling, H.G. 1972. The ascites tumor cell. In: Transport and Accumulation in Biological Systems. E.J. Harris, ed. Butterworth and Co., Ltd. London. 271-288.

Hempling, H.G., A.D. Cicoria, A.M. Dupre, and S. Thompson. 1981. State of water and electrolytes in mammalian cells during maturation and differentiation. J. Exp. Zoo. 215:259-276.

Hendil, K.B. and E.K. Hoffmann. 1974. Cell volume regulation in Ehrlich ascites tumor cells. J. Cell Physiol. 84:115-126.

Henius, G.V., P.C. Laris, and J.D. Woodburn. 1979. The preparation and properties of cytoplasts from Ehrlich ascites tumor cells. Exp. Cell Res. 121:337-345.

Herdson, P.B. and J.P. Kaltenbach. 1966. Fine structural changes in hypotonically treated Ehrlich ascites tumor cells. Exp. Cell Res. 42:74-83.

Hoffman, J.F. 1977. Ionic transport across the plasma membrane. J. Hosp. Prac. 9(10):119-127.

Hoffman, J.F. and F.M. Kregenow. 1966. The characterization of new energy dependent cation transport processes in red blood cells. Ann. N. Y. Acad. Sci. 137:566-576.

Hoffmann, E.K. 1977. Control of cell volume. In: Transport of Ions and Water in Animals. B.L. Gupta, R.B. Moreton, J.L. Oschamn, and B.J. Wall, eds. Academic Press, New York. 285-332.

Hoffmann, E.K. 1978. Regulation of selective changes in leak permeabilities of Ehrlich ascites tumor cells. In: Osmotic and Volume Regulation, Alfred Benzon Symposium XI. C.B. Jørgensen and E. Skadhauge, eds. Academic Press, New York. 397-418.

Hoffmann, E.K. and K.B. Hendil. 1976. The role of amino acids and taurine in isosmotic intracellular regulation in Ehrlich ascites mouse tumour cells. J. Comp. Physiol. 108:279-286.

Hoffmann, E.K., L.O. Simonsen, and C. Sjoholm. 1979. Membrane potential, chloride exchange, and chloride conductance in Ehrlich mouse ascites tumour cells. J. Physiol. 296:61-84.

Hogue, M.J. 1919. The effect of hypotonic and hypertonic solutions on fibroblasts of the embryonic chick heart in vitro. J. Exp. Med. 30:617-649.

Jackson, H., Jr. 1927. The histogenesis of urinary casts. Amer. J. Pathol. 3:285-288.

Jacquez, J.A. 1961. Transport and exchange diffusion of L-tryptophan in Ehrlich cells. Amer. J. Physiol. 200:1063-1068.

Jewell, S.A., G. Bellomo, H. Thor, S. Orrenius, and M.T. Smith. 1982. Bleb formation in hepatocytes during drug metabolism is caused by disturbances in thiol and calcium ion homeostasis. Science 217:1257-1258.

Johnstone, R.M. and P.G. Scholefield. 1965. The need for ions during transport and exchange diffusion of amino acids into Ehrlich ascites carcinoma cells. Biochim. Biophys. Acta 94:130-135.

Jones, R.T., R.T. Johnson, B.L. Gupta, and T.A. Hall. 1979. The quantitative measurement of electrolyte elements in nuclei of maturing erythrocytes of chick embryo using electron-probe x-ray microanalysis. J. Cell Sci. 35:67-85.

Kessel, R.G. 1968. Annulate lamellae. J. Ultrastruct. Res. Suppl. 10:1-82.

King, D.W., S.R. Paulson, N.L. Puckett, and A.T. Krebs. 1959a. Cell death. III. The effect of injury on water and electrolytes of Ehrlich tumor cells. Amer. J. Pathol. 35:835-849.

King, D.W., S.R. Paulson, N.L. Puckett, and A.T. Krebs. 1959b. Cell death. IV. The effect of injury on the entrance of vital dye in Ehrlich tumor cells. Amer. J. Pathol. 35:1067-1079.

Kitching, J.A. 1934. The physiology of contractile vacuoles. I. Osmotic relations. J. Exp. Biol. 11:364-381.

Klein, G. 1950. Use of the Ehrlich ascites tumor of mice for quantitative studies on the growth and biochemistry of neoplastic cells. Cancer 3:1052-1061.

Klein, R.L. and A.P. Breland. 1966. Active cation transport and ATP hydrolysis in Acanthamoeba Sp. Comp. Biochem. Physiol. 17:39-47.

Kleinzeller, A. 1965. The volume regulation in some animal cells. Arch. Biol. (Liege) 76: 217-232.

Kleinzeller, A. 1972. Cellular transport of water. In: Metabolic Pathways, 3rd. edition, Vol. VI. Metabolic Transport. L.E. Hokin, ed. Academic Press, New York. 91-131.

Knulton, S., D. Jackson, J.M. Graham, K.J. Micklem, and C.A. Pasternak. 1976. Microvilli and cell swelling. Nature 262:52-54.

Kregenow, F.M. 1971. The response of duck erythrocytes to nonhemolytic hypotonic media. Evidence for a volume-controlling mechanism. J. Gen. Physiol. 58:372-395.

Kregenow, F.M. 1973. The response of duck erythrocytes to norepinephrine and an elevated extracellular potassium. J. Gen. Physiol. 61:509-527.

Kregenow, F.M. 1974. Functional separation of Na-K exchange pump from the volume controlling mechanism in enlarged duck red cells. J. Gen. Physiol. 64:393-412.

Kregenow, F.M. 1981. Osmoregulatory salt transporting mechanisms: control of cell volume in anisotonic media. Ann. Rev. Physiol. 43:493-505.

Kregenow, F.M., D.E. Robbie, and J. Orloff. 1976. Effect of norepinephrine and hypertonicity on K influx and cyclic AMP in duck erythrocytes. Amer. J. Physiol. 231:306-312.

Kreisberg, J.I., J.W. Mills, J.A. Jarrell, C.A. Rabito, and A. Leaf. 1980. Protection of cultured renal tubular epithelial cells from anoxic cell swelling and cell death. Proc. Natl Acad. Sci. 77:5445-5447.

Kristensen, Be.I., L.O. Simonsen, and L. Pape. 1973. Actin-like filaments in Ehrlich ascites tumor cells and their reaction with heavy meromyosin. Virchows Archiv. (Zellpathol.) 13:103-112.

Laiho, K.U., J.D. Shelburne, and B.F. Trump. 1971. Observations on cell volume, ultrastructure, mitochondrial conformation and vital-dye uptake in Ehrlich ascites tumor cells: effects of inhibiting energy production and function of the plasma membrane. Amer. J. Pathol. 65:203-230.

Laiho, K.U. and B.F. Trump. 1974. The relationship between cell viability and changes in mitochondrial ultrastructure, cellular ATP, ion and water content following injury of Ehrlich ascites tumor cells. Virchows Arch. (Zellpathol.) 15:267-277.

Laiho, K.U. and B.F. Trump. 1975. Studies on the pathogenesis of cell injury. Effects of inhibitors of metabolism and membrane function on the mitochondria of Ehrlich ascites tumor cells. Lab. Invest. 32(2):163-183.

Lala, P.K. 1972. Evaluation of the mode of cell death in Ehrlich ascites tumor. Cancer 29:261-266.

Lang, M.A. and H. Gainer. 1969. Volume control by muscle fibers of the blue crab. J. Gen. Physiol. 53:323-341.

Lange, R. 1964. The osmotic adjustment in the echinoderm, Strongylocerotus droebachiensis. Comp. Biochem. Physiol. 13:205-216.

Lange, R. and K. Fugelli. 1965. The osmotic adjustment in the euryhaline teleosts, the flounder, Pleuronectes flesus L. and the three-spined stickleback, Gasterosteus aculeatus L. Comp. Biochem. Physiol. 15:283-292.

Laris, P.C., M. Bootman, H.A. Pershadsingh, and R.M. Johnstone. 1978. The influence of cellular amino acids and the Na^+:K^+, pump on the membrane potential of the Ehrlich ascites tumor cell. 1978. Biochim. Biophys. Acta 512:397-414.

Laris, P.C. and G.V. Henius. 1982. Influence of glucose on Ehrlich cell volume, ion transport, and membrane potential. Amer. J. Physiol. 242:c326-332.

Lassen, U.V., A.M.T. Nielson, L. Pape, and L.O. Simonsen. 1971. The membrane potential of Ehrlich ascites tumor cells microelectrode measurements and their ciritical evaluation. J. Membrane Biol. 6:269-288.

Leaf, A. 1956. On the mechanism of fluid exchange of tissues in vitro. Biochem. J. 62:241-248.

Leaf, A. 1959. Maintenance of concentration gradients and regulation of cell volume. Ann. N. Y. Acad. Sci. 72:396-404.

Leaf, A. 1970. Regulation of intracellular fluid volume and disease. Amer. J. Med. 49(3):291-295.

Lettré, H., M. Albrecht, and R. Lettre. 1951. Verhalten von Fibroblasten unter aeroben und anaeroben Bedingungen. Naturwissenshaften 38:504-505.

Lettré, R., N. Paweletz, D. Werner, and C. Granzow. 1972. Sublines of the Ehrlich-Lettre mouse ascites tumour. A new tool for experimental cell research. Naturwissenschaften 59:59-63.

Levinson, C. 1967. Calcium exchange in Ehrlich mouse ascites tumor cells. Biochim. Biophys. Acta 135:921-926.

Lewis, M.R. 1923. Reversible gelation in living cells. Bull. Johns Hopkins Hosp. 34:373-379.

Lucké, B. and M. McCutcheon. 1932. The living cell as an osmotic system and its permeability to water. Physiol. Rev. 12:68-139.

Macknight, A.D.C. 1968. Water and electrolyte contents of rat renal cortical slices incubated in potassium-free media and media containing ouabain. Biochim. Biophys. Acta 150:263-270.

Macknight, A.D.C., M.M. Civan, and A. Leaf. 1975. Some effects of ouabain on cellular ions and water in epithelial cells of toad urinary bladder. J. Membrane Biol. 20:387-401.

Macknight, A.D.C. and A. Leaf. 1977. Regulation of cellular volume. Physiol. Rev. 57:510-573.

MacRobbie, E.A.C. and H.H. Ussing. 1961. Osmotic behavior of the epithelial cells of frog skin. Acta Physiol. Scand. 53:348-365.

Maffly, R.H. and A. Leaf. 1959. The potential of water in mammalian tissues. J. Gen. Physiol. 42:1257-1275.

Maizels, M., M. Remington, and R. Truscoe. 1958a. Data for the calculation of the rate coefficients of sodium transfer by mouse ascites tumour cells. J. Physiol. 140:48-60.

Maizels, M., M. Remington, and R. Truscoe. 1958b. The effects of certain physical factors and of the cardiac glycosides on sodium transfer by mouse ascites tumour cells. J. Physiol. 140:61-79.

Malenkov, A.G., V.A. Artamonova, M.L. Kakushkina, G.G. Slivinsky, and V.L. Voejkov. 1974. The mechanism of the autonomy of division of ascitic tumor cells. Int. J. Cancer 14(5):691-697.

Matsudaira, P.T. and D.R. Burgess. 1979. Identification and organization of the components in the isolated microvillus cytoskeleton. J. Cell Biol. 83:667-673.

Mayhew, E., J.P. Harlos, and R.L. Juliano. 1973. The effect of polycations on cell membrane stability and transport processes. J. Membrane Biol. 14(3):213-228.

Mayhew, E., and C. Levinson. 1968. Reversibility of ouabain induced inhibition of cell division and cation transport in Ehrlich ascites tumor cells. J. Cell Physiol. 72:73-75.

McIver, D.J.L. and A.D.C. Macknight. 1974. Extracellular space in some isolated tissues. J. Physiol. 239:31-49.

Medzihradsky, F. and M.J. Marks. 1975. Measures of viability in isolated cells. Biochem. Med. 13(2):164-177.

Mehrishi, J.N. 1972. Surface molecular components of Ehrlich ascites tumour cells. Blut. 24(6): 388-397.

Mitchell, R.F. 1971. A study of the cytoplasmic matrix of ascites tumour cells. Exp. Cell Res. 66:137-144.

Morrill, G.A., H.R. Kaback, and E. Robbins. 1964. Effect of calcium on intracellular sodium and potassium concentrations in plant and animal cells. Nature 204:641-642.

Mudge, G.H. 1951. Studies on potassium accumulation by rabbit kidney slices: effect of metabolic activity. Amer. J. Physiol. 165:113-127.

Nellans, H.N., R.A. Frizzell, and S.G. Schultz. 1974. Brush border processes and transepithelial Na and Cl transport by rabbit ileum. Amer. J. Physiol. 226:1131-1141.

Neville, M.C. and D.K. Nordstrom. 1973. The effect of external K^+ concentration on the steady level of cellular K^+ and Na^+ in Ehrlich ascites cells. Biophys. J. 13:229a (Abstr.)

Nirenberg, M.W. 1959. A biochemical characteristic of ascites tumor cells. J. Biol. Chem. 234:3088-3093.

Opie, E.L. 1949. The movement of water in tissues removed from the body and its relation to movement of water during life. J. Exp. Med. 89:185-208.

Owellen, R.J., D.W. Donigian, C.A. Hartke, R.M. Dickerson, and M.J. Kuhar. 1974. The binding of vinblastine to tubulin and to particulate fractions of mammalian brain. Cancer Res. 34 (3):3180-3186.

Palfrey, H.C., P.W. Feit, and P. Greengard. 1980. cAMP-stimulated cation cotransport in avian erythrocytes: inhibition by "loop" diuretics. Amer. J. Physiol. 238(3):C139-C148.

Panniers, L.R. and M.J. Clemens. 1981. Inhibition of cell division by interferon: changes in cell cycle characteristics and in morphology of Ehrlich ascites tumour cells in culture. J. Cell Sci. 48:259-279.

Pappenheimer, A.M. 1917. Experimental studies upon lymphocytes. I. The reactions of lymphocytes under various experimental conditions. J. Exp. Med. 25:633-650.

Parker, J.C. 1973. Dog red blood cells. Adjustment of density in vivo. J. Gen. Physiol. 61:146-157.

Pasternak, C.A. 1976. Surface membranes during the cell cycle. TIBS 148-151.

Penttila, A. and B.F. Trump. 1974a. Extracellular acidosis protects Ehrlich ascites tumor cells and rat renal cortex against anoxic injury. Science 185:277-278.

Penttila, A. and B.F. Trump. 1974b. Protective effect of acidosis against cell injury. Fed. Proc. 33(1):639 (Abstr.).

Penttila, A. and B.F. Trump. 1975a. Studies on the modification of the cellular response to injury. II. Electron microscopic studies on the protective effect of acidosis on anoxic injury of Ehrlich ascites tumor cells. Virchows Archiv. (Cell Pathol.) 18(1):1-16.

Penttila, A. and B.F. Trump. 1975b. Studies on the modification of the cellular response to injury. III. Electron microscopic studies on the protective effect of acidosis on p-chloromercuribenzene sulfonic acid- (PCMBS) induced injury of Ehrlich ascites tumor cells. Virchows Arch. (Cell Pathol.) 18(2):17-34.

Penttila, A. and B.F. Trump. 1975c. Studies on the modification of the cellular response to injury. I. Protective effect of acidosis on p-chloromercuribenzene sulfonic acid-induced injury of Ehrlich ascites tumor cells. Lab. Invest. 32(6):690-695.

Persson, B.E. and K.R. Spring. 1982. Gallbladder epithelial cell hydraulic water permeability and volume regulation. J. Gen. Physiol. 70(3):481-505.

Pietrzyk, C. and E. Heinz. 1972. Some observations on the non homogeneous distribution inside the Ehrlich cell. In: Sodium-linked transport of organic solutes. E. Heinz, ed., Springer-Verlag, Berlin. 84-90.

Pietrzyk, C. and E. Heinz. 1974. The sequestration of Na^+, K^+, and Cl^- in the cellular nucleus and its energetic consequences for the gradient hypothesis of amino acid transport in Ehrlich cells. Biochim. Biophys. Acta 352:397-411.

Ponder, E. and G. Saslow. 1931. The measurement of red cell volume. III. Alterations of cell volume in extremely hypotonic solutions. J. Physiol. 73:267-296.

Porter, K.R., T.T. Puck, A.W. Hsie, and D. Kelley. 1974. An electron microscope study of the effects of dibutyryl cyclic AMP on Chinese hamster ovary cells. Cell 2:145-162.

Porter, K.R., and J.B. Tucker, 1981. The ground substance of the living cell. Sci. Amer. 244(3):57-67.

Post, R.L., C.R. Merritt, C.R. Kinsolving and C.D. Albright. 1960. Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte. J. Biol. Chem. 235(6):1796-1802.

Potashner, S.J. and R.M. Johnstone. 1971. Cation gradients, ATP, and amino acid accumulation in Ehrlich ascites cells. Biochim. Biophys. Acta 233:91-103.

Price, Z.H. 1967. The micromorphology of zeiotic blebs in cultured human epithelial (HEp) cells. Exp. Cell Res. 48:82-92.

Prusch, R.D. 1977. Protozoan osmotic and ionic regulation. In: Transport of Ions and Water in Animals. B.L. Gupta, R.B. Moreton, J.L. Oschman, and B.J. wall, eds. Academic Press, New York. 363-377.

Rand, R.P. 1964. Mechanical properties of the red cell membrane. II. Viscoelastic breakdown of the membrane. Biophys. J. 4:303-316.

Rand, R.P. and A.C. Burton. 1964. Mechanical properties of the red cell membrane. I. Membrane stiffness and intracellular pressure. Biophys. J. 4:115-135.

Reuber, J.P., E. Lopez, P.W. Brandt, and H. Grundfest. 1963. Muscle: volume changes in isolated single fibers. Science 142:246-248.

Riddick, D.H., F.M. Kregenow, and J. Orloff. 1971. Effect of norepinephrine and dibutyryl cyclic adenosine monophosphate on cation transport in duck erythrocytes. J. Gen. Physiol. 57:752-766.

Robinson, J.R. 1950. Osmoregulation in surviving slices from the kidneys of adult rats. Proc. Roy. Soc., London Series B 137:378-402.

Robinson, J.R. 1953. The active transport of water in living systems. Biol. Rev. 28:158-194.

Rorive, G. and R. Gilles. 1979. Intracellular inorganic effectors. In: Mechanisms of Osmoregulation in Animals: Maintenance of Cell Volume. R. Gilles, ed. Part B (13). Wiley, New York. 83-108.

Rosenberg, H.M., B.B. Shank, and E.C. Gregg. 1972. Volume changes of mammalian cells subjected to hypotonic solutions in vitro: evidence for the requirement of a sodium pump for the shrinking phase. J. Cell Physiol. 80(1):23-32.

Roti Roti, L.W. and A. Rothstein. 1973. Adaptation of mouse leukemic cells (L5178Y) to anisotonic media. I. Cell volume regulation. Exp. Cell Res. 79:295-310.

Rubin, R.W. and L.P. Everhart. 1973. The effect of cell-to-cell contact on the surface morphology of Chinese hamster ovary cells. J. Cell Biol. 57:837-844.

Schafer, J.A. 1977. A reassessment of decreased amino acid accumulation by Ehrlich ascites tumor cells in the presence of metabolic inhibitors. J. Gen. Physiol. 69:681-704.

Schmid-Schönbein, G.W., Y.Y. Shih, and S. Chien. 1980. Morphometry of human leukocytes. Blood. 56(5):866-875.

Schmidt, W.F. and T.J. McManus. 1977. Ouabain-insensitive salt and water movements in duck red cells. II. Norepinephrine stimulation of sodium plus potassium cotransport. J. Gen. Physiol. 70:81-97.

Schmidt-Nielsen, B. 1975. Comparative physiology of cellular ion and volume regulation. J. Exp. Zool. 194:207-220.

Schoffeniels, E. 1967. Cellular aspects of membrane permeability. Protoplasma 63:66-69.

Seely, J.F. and J.H. Dirks. 1977. Site of action of diuretic drugs. Kidney Int. 11:1-8.

Selby, C.C., J.J. Biesele, and C.E. Grey. 1956. Electron microscope studies of ascites tumor cells. Ann. N. Y. Acad. Sci. 63:748-773.

Shank, B.B., H.M. Rosenberg, and C. Horowitz. 1973. Ionic basis of volume regulation in mammalian cells following osmotic shock. J. Cell Physiol. 82:257-266.

Shear, M.J. 1935. Chemical studies on tumor tissue: II. The effect of protein on the swelling of normal and tumor cells of mice in vitro. Amer. J. Cancer 23:771-783.

Siebens, A.W. and F.M. Kregenow. 1978. Volume regulatory responses of salamander red cells incubated in anisotonic media--effect of amiloride. Physiologist 21(4):110 (Abstr.)

Sigler, K. and K. Janacek. 1971. The effect of non-electrolyte osmolarity on frog oocytes. II. Intracellular potential. Biochim. Biophy. Acta 241:539-546.

Simonsen, L.O. and A.M.T. Nielsen. 1971. Exchangeability of chloride in Ehrlich ascites tumor cells. Biochim. Biophys. Acta 241:522-527.

Skou, J.C. 1957. The influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochim. Biophys. Acta 23:394-401.

Skou, J.C. 1965. Enzymatic basis for active transport of Na^+ and K^+ across the cell membrane. Physiol. Rev. 45:596-617.

Smith, T.C. and R. Adams. 1977. Intracellular compartmentation of Na^+, K^+, and Cl^- in the Ehrlich ascites tumor cell: correlation with the membrane potential. J. Membrane Biol. 35:57-74.

Smith, T.C., T.M. Mikiten, and C. Levinson. 1972. The effect of multivalent cations on the membrane potential of the Ehrlich ascites tumor cell. J. Cell Physiol. 79:117-126.

Smith, T.C. and S.C. Robinson. 1981. Variable coupling of active Na^+ and K^+ transport in Ehrlich ascites tumor cells: regulation by external Na^+ and K^+. J. Cell Physiol. 106:407-418.

Smith, T.C. and K.D. Vernon. 1979. Correlation of the effect of Ca^{+2} on Na^+ amd K^+ permeability and membrane potential of Ehrlich ascites tumor cells. J. Cell Physiol. 98:359-370.

Spector, A.A. 1975. Fatty acid metabolism in tumors. Prog. Biochem. Pharmacol. 10:42-75.

Speth, V., H.C. Bauer, and G. Brunner. 1981. Changes of the internal organization of the plasma membrane correlated to the regeneration potency of the cell. Biochim. Biophys. Acta 649:113-120.

Spring, K.R. and B.E. Persson. 1981. Quantitative light microscopy and epithelial function. In: Epithelial Ion and Water transport. A.D.C. Macknight and J.P. Leader, eds. Raven Press, New York.

Sutherland, E.W. and G.A. Robison. 1966. Metabolic effects of catecholamines. A. The role of cyclic 3'5'-AMP in response to catecholamines and other hormones. Pharmacol. Rev. 18:145-161.

Sweadner, K.J. and S.M. Goldin. 1980. Active transport of sodium and potassium ions: mechanism, function, and regulation. N. Eng. J. Med. 302(14):777-783.

Swift, J. and E. Rasch. 1958. Studies on electron microscope cytochemistry. Scient. Instr. News 3:1-9.

Thomas, R.C. 1977. The role of bicarbonate, chloride and sodium ions in the regulation of intracellular pH in snail neurones. J. Physiol. 273:317-338.

Tosteson, D.C. 1964. Regulation of cell volume by sodium and potassium transport. In: The Cellular Functions of Membrane Transport. J.F. Hoffman, ed. Prentice-Hall, Inc., Englewood Cliffs, New Jersey. 3-22.

Tosteson, D.C. and J.F. Hoffman. 1958. Cation transport in high and low potassium sheep red cells. J. Cell and Comp. Physiol. 52:191 (Abstr.).

Tosteson, D.C. and J.F. Hoffman. 1960. Regulation of cell volume by active cation transport in high and low potassium sheep red cells. J. Gen. Physiol. 44:169-194.

Trump, B.F. and F.L. Ginn. 1968. Studies of cellular injury in isolated flounder tubules. II. Cellular swelling in high potassium media. Lab. Invest. 18:341-351.

Trump, B.F., A. Penttila, and I.K. Berezesky. 1979a. Studies on cell surface conformation following injury. I. Scanning and transmission electron microscopy of cell surface changes following p-chloromercuribenzene sulfonic acid (PCMBS) induced injury of Ehrlich ascites tumor cells. Virchows Arch. (Cell Pathol.) 29(4):281-296.

Trump, B.F., A. Penttila, and I.K. Berezesky. 1979b. Studies on cell surface conformation following injury. II. Scanning and transmission electron microscopy of cell surface changes following anoxic injury in Ehrlich ascites tumor cells. Virchows Arch. (Cell Pathol.) 29(4):297-307.

Ussing, H.H. 1978. Concluding remarks. In: Osmotic and Volume Regulation, Alfred Benzon Symposium XI. C.B. Jorgensen and E. Skadhauge, eds. Academic Press, New York.

Vislie, T. and K. Fugelli. 1975. Cell volume regulation in flounder (Platichthys flesus) heart muscle accompanying an alteration in plasma osmolality. Comp. Biochem. Physiol. 52A:415-418.

Wallach, D.F.H. and V.B. Kamat. 1966. The contribution of sialic acid to the surface charge of fragments of plasma membrane and endoplasmic reticulum. J. Cell Biol. 30:660-663.

Wallach, D.F.H., V.B. Kamat, and M.H. Gail. 1966. Physicochemical differences between fragments of plasma membrane and endoplasmic reticulum. J. Cell Biology 30:601-621.

Wallach, D.F.H. and V. Vlahovic. 1967. Immumological cross-reactivity between plasma membrane and endoplasmic reticulum of Ehrlich ascites carcinoma. Nature 216:182.

Weed, R.I. and M. Bessis. 1973. The discocyte-stomatocyte equilibrium of normal and pathologic red cells. Blood 41:471-475.

Weibel, E.R. 1963. Principles and methods for the morphometric study of the lung and other organs. Lab. Invest. 12:131-155.

Weibel, E.R. 1973. Sterelogical techniques for electron microscopic morphometry. In: Principles and Techniques of Electron Microscopy. Volume 3 M.A. Hayat, ed. Van Nostrand Rindhold Co., New York. 237-296.

Weihing, R.R. 1976. Occurrence of microfilaments in non-muscle cells and tissues. In: Biological Handbooks, Cell Biology Volume I. P.L. Altman and D.D. Katz, eds. FASEB Publ., Bethesda, Maryland.

Welling, D.J., L.W. Welling, and J.J. Hill. 1978. Phenomenological model relating cell shape to water reabsorption proximal nephron. Am. J. Physiol. 234(4):F308-F317.

Whittembury, G. and J.J. Grantham. 1976. Cellular aspects of renal sodium transport and cell volume regulation. Kidney Int. 9:103-120.

Wikstrom, B., G. Ronquist, and G. Agren. 1971. Formation of cyclic adenosine-3',5'-monophosphate (cyclic AMP) by a crude membraneous fraction of Ehrlich ascites tumour cells. Acta Chem. Scand. 25(10):3785-3790.

Wiley, J.S. and R.A. Cooper. 1974. A furosemide-sensitive cotransport of sodium plus potassium in the human red cell. J. Clin. Invest. 53:745-755.

Wilson, T.H. 1954. Ionic permeability and osmotic swelling of cells. Science 120:104-105.

Wischnitzer, S. 1970. The annulate lamellae. Int. Rev. Cytol. 27:65-100.

Wood, J.G., D.H. Jean, J.N. Whitaker, B.J. McLaughlin, and R.W. Albers. 1977. Immunocytochemical localization of the sodium, potassium activated ATPase in knifefish brain. J. Neurocytol. 6:571-581.

Yancey, P.H., M.E. Clark, S.C. Hand, R.D. Bowlus, and G.N. Somero. 1982. Living with water stress: evolution of osmolyte systems. Science 217:1214-1222.

Yasuzumi, G. and R. Sugihara. 1958. A comparative electron microscopic study on Ehrlich ascites tumor cells, Yoshida sarcoma cells and human cancerous peritonitis ascites cells. Cancer Res. 18:1167-1175.

Zollinger, H.U. 1948. Cytological studies with the phase microscope. I. The formation of "blisters" on cells in suspension (potocytosis), with observations on the nature of the cellular membrane. Amer. J. Pathol. 24:545-558.

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA IN BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Elizabeth Haughie Wilkerson

Major Subject Physiology and Biophysics

Title of Dissertation Effect of Hypotonic Media on Ehrlich Ascites Tumor Cells: Correlation of Morphologic Changes with Ion and Water Movements

Dissertation Committee:

_____, Chairman

_____

_____

_____

_____

_____

Director of Graduate Program _____

Dean, UAB Graduate School _____

Date 6/3/83

PS-1428