Please return proofs to Scanning Microscopy Intl. Dr Om Johari P.O. Box 66507 AMF O'Hare, IL 60666-05 USA

Scanning Microscopy, Vol 12, No. 1, 1998 (Pages )
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/98$5.00+.25

# BASIC BIOLOGICAL RESEARCH WITH THE STRIATED MUSCLE BY USING CRYOTECHNIQUES AND ELECTRON MICROSCOPY

L. Edelmann

Medizinische Biologie, Fachbereich Theoretische Medizin, Universität des Saarlandes, D-66421 Homburg/Saar, Germany
Telephone number: ++49-6841-166254/Fax number: ++49-6841-166256

(Received for publication May 10, 1997 and in revised form October 7, 1997)

## Abstract

Several basic mechanisms underlying living phenomena are not really understood. Unequivocal interpretations of data concerning the following phenomena - to name but a few - are missing: cellular accumulation of potassium, cellular exclusion of sodium, cell volume regulation, shape change of cells (e.g., of muscle cells during contraction), electrical potential differences between inside and outside of living cells. The theoretical treatment of these phenomena as found in all current textbooks is based on the membrane-pump theory (MPT) with the following essential features: The bulk of the main cellular cation $K^+$ is freely dissolved in free cellular water and membrane-situated pumps are responsible for the high level of $K^+$ and the low level of $Na^+$ found in virtually all living cells. On the other hand, the above mentioned phenomena are explained by the association-induction hypothesis (AIH) without the proposal of membrane-situated pumps and with the postulation of selective $K^+$ adsorption to cellular proteins and of a specific cell water structure which has a low solvency for $Na^+$ and other solutes. Experimental findings are reviewed which contradict the MPT and support the AIH. In addition, electron microscopic experiments with cryoprocessed striated muscle are reviewed which establish cellular $K^+$ binding (adsorption) and a cellular water structure which is different from that of normal free water. Cryoexperiments with the striated muscle and model systems are proposed which may help to obtain further information on the specific interactions between proteins, ions, and water in living cells.

**Key Words:** Membrane-pump theory, association-induction hypothesis, alkali-metal ions, ion adsorption, water structure, muscle, model systems, muscle contraction, cryotechniques, vitrification, freeze-drying.

## Introduction

According to virtually all textbooks the basic mechanisms underlying living phenomena are described by the membrane pump theory (MPT). Physiological properties of living cells such as selective solute accumulation and exclusion, selective permeability, maintenance and change of cell volume, and cellular electrical potentials are described by properties of the cell membrane. In particular, selective solute accumulation and exclusion require energy consuming (active) transport mechanisms (pumps) which are localized in the cell membrane. An alternative view is provided by the association-induction-hypothesis (AIH) of Ling (Ling, 1962, 1984, 1992a, 1994) which explains living phenomena as the result of the cohesive interaction among the cells three major components: proteins, ions and water. Solute accumulation and exclusion are caused by specific properties of cellular proteins and cellular water. Membrane-situated pumps do not exist in unifacial cells like muscle, nerve, erythrocyte. (Note that active transport across bifacial cell systems like frog skin or intestinal epithelium has never been disputed; for the theoretical treatment of such active transport phenomena according to the AIH see Ling, 1984, Chapter 17; Ling, 1990).

Although the controversy between these different schools of thought has been going on for many decades it is not described in textbooks or review articles, the controversy is simply ignored by the scientific establishment. It is the purpose of this paper to show by means of a few experimental facts that membrane pumps are unproved postulations and that progress in understanding of basic biological phenomena is expected by investigating the specific interactions between cellular proteins, ions, and water as suggested by the AIH. It is shown that in particular electron microscopy of the striated muscle has already confirmed major predictions of the AIH and may be further used to explore properties of cellular proteins and water.

## Living Phenomena According to MPT and AIH

A model of the living cell has to explain the following facts (Ling, 1992a, p. 31): In mass, the largest component of living cells is water, the second largest is protein. In number, the most abundant molecule is water, the second most abundant is $K^+$. By and large, the external environment of cells is a salt solution containing water as its largest component and NaCl as its next largest component. Water and both $Na^+$ and $K^+$ are continuously in exchange between the cell interior and the external medium.

These facts are explained in the MPT as follows. The bulk of cellular water is free water in which most of cellular ions are freely dissolved. The asymmetric $K^+/Na^+$ distribution is due to active and passive transport mechanisms localized in the cell membrane. Essential is the existence of an energy consuming $Na^+$ pump which is responsible for a continuous active transport of cellular $Na^+$ to the outside solution. These features are basic to the description of many other cellular phenomena. E.g., cell volume regulation is determined primarily by the osmotic pressure exerted by solutes that are freely dissolved within the free cellular water. $K^+$ - the main cellular cation - is thought to be the main solute that helps to balance the osmotic pressure of the cell interior and of the extracellular medium. According to the MPT an intact cell membrane is essential for the maintenance of cell volume. The electrical potentials of living cells are membrane diffusion potentials which can be described by the extra- and intracellular concentrations and the membrane permeabilities of certain ions. For instance, the resting potential of nerve and muscle cells is in first approximation a $K^+$ diffusion potential as described by the Hodgkin-Katz theory (Hodgkin and Katz, 1949). In analogy to the $Na^+$ pump concept a variety of membrane-situated pumps explain why many different permeable substances are accumulated in or partly excluded from the free cellular water.

Based on statistical mechanics the AIH is an equilibrium theory which describes phenomena of living cells without membrane pumps. The facts presented in the first section of this chapter are explained by two mechanisms (Ling, 1984, p. 375):

(M1) "The bulk of cellular water exists in a state of polarized multilayers; in this state water tends to exclude solutes and does so to variable degrees depending on the size and complexity of the solute. This provides the mechanism for the normal exclusion of $Na^+$ from most cells."

(M2) "Solutes are accumulated by the cell if they are adsorbed onto macromolecules within the cell; for example, cations are adsorbed onto fixed carboxyl groups and sugars onto hydrogen bonding groups of proteins. This provides the mechanism for the normal accumulation of $K^+$ by most cells."

In the AIH the control of the reversible interactions of proteins with water and ions (as expressed by M1 and M2) is provided by a third mechanism:

(M3) "The polypeptide chain is especially well suited for the induction of electron distribution changes from one side chain to another. This underlies the interaction between sites that adsorb solutes, permitting them to function in a cooperative manner, and it underlies the ability of cardinal adsorbents (e.g., ATP, hormones, drugs) to affect a large number of sites in an allosteric manner."

Based on these three concepts the AIH is a general theory which can explain living phenomena in a consistent manner. These explanations differ considerably from the explanations given by the MPT. For example, cell volume is determined by the amount of polarized cellular water, the bulk of cellular $K^+$ is adsorbed and hence osmotically inactive. Cellular electrical potentials are seen as phase boundary potentials between the cell surface and the extracellular phase and have no direct relation to ionic permeabilities. The electrical potential is determined by the density and nature of the ionic groups on the macromolecules of the cell surface.

As mentioned in the Introduction living phenomena are described in all textbooks of life sciences by means of the MPT. The main reason for ignoring the AIH is probably the strong conviction of most scientists that the $Na^+$ pump and other pumps have been proven and that therefore a model without energy consuming membrane pumps cannot be correct. However, if the following experimental results are taken seriously one must doubt the pump concept.

In 1962 Ling presented the results of a three year long inquiry into the feasibility of the postulated $Na^+$ pump from a thermodynamic standpoint (Ling, 1962). He compared the minimum energy need of the postulated $Na^+$ pump in frog muscle cells for a recorded period of time with the maximum energy available to these cells during the same period of time. The experiments showed that the minimum energy need of the postulated $Na^+$ pump is from 1500 % to 3000 % of the maximum available energy. Disparities of this magnitude are decisive by any standard and until now there has been no challenge in print against the experiments nor the conclusions of Ling. The essence of these findings have been confirmed twice (Jones, 1965; Minkoff and Damadian, 1973).

In addition, experiments designed to prove

directly the existence of pumps failed. The perfused squid axon contains functioning $Na^+$,$K^+$-ATPase - the postulated ion pump (Glynn and Karlish, 1975) - and is able to increase $Na^+$ efflux by addition of ATP, and the efflux is sensitive to ouabain; however, a net $Na^+$ efflux against an electrochemical gradient could not be observed (for review see [Ling, 1984] p. 127). Similar results were obtained with non-leaky "white ghosts" obtained from red blood cells. These ghosts are not able to accumulate $K^+$ or extrude $Na^+$ despite the fact that they contain normally functioning $Na^+$,$K^+$-ATPase (Ling and Tucker, 1983)). The claim that reconstituted purified phospholipid-ATPase vesicles pump $Na^+$ has been analyzed in a detailed study by Ling and Negendank (1980); they came to the conclusion that ATP did not actually cause a net gain of $Na^+$ by these vesicles and that the results can be better explained by the AIH. The criticism of Ling and Negendank has not been refuted in print.

Taken together, the $Na^+$ pump has not been proven directly and - since the energy argument apparently cannot be refuted - one must conclude that the $Na^+$ pump does not exist. On the other hand, those who still believe in pumps are confronted with the fact that the molecular mechanism for the working of the pump is unknown (Glynn and Karlish, 1975, p. 13). That means: all living phenomena which are explained by means of the pump concept are not really understood.

Concerning the AIH one may ask: Why should we consider and adopt a new working hypothesis? Are the concepts underlying the AIH so general and unspecific that they would fit anything and are therefore of little value or, are these concepts experimentally verified? Has the AIH any predictive value? Can it explain phenomena which are explained by the MPT as well as phenomena which cannot be explained by the MPT? These questions have been discussed in detail by Ling (1992a) and the latter two questions have been answered positively. It must be noted that at the time of the first postulation of the concepts underlying the AIH very little experimental confirmation was available. In the meanwhile the experimental verification of the basic principles is in my opinion complete and many predictions have been confirmed by experimental testing. In the following I mention a few key experiments which clearly demand future consideration of the AIH.

In 1965 it was postulated that the bulk of cellular water is polarized in multilayers (Ling, 1965). Ling and Negendank (1970) studied the water uptake of frog muscles exposed not to solutions but to water vapors of known relative vapor pressure. They found that the water content of muscle follows the Bradley isotherm (Bradley, 1936) which was derived under the assumption that a substance with a large dipole moment (like water) is adsorbed in multilayers onto appropriate polar surfaces (like certain proteins). The AIH postulates that water polarized in multilayers has a reduced size-dependent solvency for solutes (Ling, 1984). Ling and co-workers have shown that water of so called *"extrovert"* solutions (certain polymers, certain proteins) and of living muscle cells exhibit the postulated size-dependent reduced solvency (for reviews see Ling, 1992a,b).

According to the multilayer theory of cell water and of solutions of *extroverts* the water molecules suffer motional restriction, in particular rotational motional restriction. This prediction has been confirmed by quasi-elastic neutron scattering (Trantham *et al.*, 1984; Rorschach *et al.*, 1987; see also Negendank, 1986).

According to the AIH cell volume regulation is primarily due to interactions between cellular proteins ions and water. Experimental testing shows that the maintenance of normal muscle cell volume, its swelling in hypotonic solutions as well as in concentrated KCl solutions are indifferent to the presence of an intact cell membrane (Ling and Walton, 1976). The finding is incompatible with the explanation of cell volume regulation given by the MPT. Note that this finding and others which are not compatible with the MPT are as a rule ignored in review articles on cell volume regulation (see e.g., Hoffmann and Dunham, 1995).

The AIH postulates that $K^+$ accumulation and $Na^+$ exclusion by living cells is due to cytoplasmic properties (specific $K^+$ adsorption to macromolecules and reduced $Na^+$ solvency in cellular water) and not due to membrane pumps. Both phenomena have been verified by direct exposure of muscle cytoplasm to Ringer's solution (effectively open ended cell [EMOC] preparation, Ling, 1978). With the aid of the EMOC preparation it could also be demonstrated that ouabain changes the cytoplasmic $K^+/Na^+$ distribution without a functional cell membrane (hence without using the postulated $Na^+$ pump). In addition, a complex binding of cellular $K^+$ has been established by analysis of the X-ray absorption edge fine structure of $K^+$ in frog blood cells (Huang *et al.*, 1979).

A prediction of the AIH is that $K^+$ accumulation in muscle follows the distribution of $\beta$- and $\gamma$-carboxyl groups fixed to cellular proteins. This prediction has been confirmed by light and electron microscopic studies (see below).

The AIH predicts that the electrical potential of cells is dependent on ion adsorption at the cell surface and not on ion permeabilities. This prediction has been

confirmed with guinea pig heart muscle cells by using $K^+$, $Rb^+$, and $Cs^+$ ions (Edelmann, 1973).

One notes that without doubting the membrane pump concept and without adopting a new working hypothesis the described experiments (and many others) would not have been performed. The results of these efforts indicate that the goal of understanding fundamental living phenomena requires a new focus of interest towards the specific interactions between cellular proteins, ions and water. In the following it is shown that electron microscopy of the striated muscle can be used as an important tool for studying these interactions.

## Water and Ions in the Striated Muscle: Problems for Cryopreparation and Electron Microscopy

In the first half of this century different, seemingly critical, experiments with the striated muscle played a major role in adopting the concept that the main cellular cation, $K^+$, is freely dissolved in free cell water (MPT, see above):

(1) Based on the finding that urea distributes equally between muscle cell water and the external medium the Nobel Laureate Hill concluded that no nonsolvent water exists in frog muscle (Hill, 1930). In addition, Hill and Kupalov (1930) found the vapor pressure of frog muscle equal to that of an isotonic 0.1 M NaCl solution. These studies led to the conclusion that only free water exists in the muscle cells, and since the total ionic concentration in the cell is about 0.1 M and because $K^+$ constitutes the bulk of the cations it follows that cellular $K^+$ (and anions) must be free in order to balance the osmotic activity of free $Na^+$ and free $Cl^-$ in the free extracellular water.

(2) Microincineration studies led Macallum (1905) to conclude that $K^+$ is preferentially accumulated in the A band of the striated muscle. This idea has been refuted by Gersh (1938) who used improved and superior histochemical precipitation methods for ion localization. He found no preferential accumulation of $K^+$ in the A band.

(3) Studies of isolated muscle proteins and of other isolated proteins failed to show any significant degree of selective $K^+$ binding (Lewis and Saroff, 1957; Carr, 1956).

Despite these results a minority always doubted the concept of free cellular $K^+$ and free cellular water (for reviews see Troschin, 1958, 1966; Ling, 1962; Ernst, 1963). But it was mainly the energy argument of Ling (see above) and a prediction concerning the subcellular distribution of $K^+$ in the striated muscle which led to a reinvestigation of the ion and water problems with newly designed electron microscopic methods. Before discussing these efforts a main problem of electron microscopy should be mentioned.

The purpose of electron microscopic investigation of biological material is to understand how living cells are constructed and how they function. However, the watery biological material in its natural state is not suited for investigation in the vacuum of an electron microscope (EM). It must be stabilized somehow before it can be examined in the EM. Now, if we are interested in the interactions between the main components of living cells - namely proteins, water and ions - the conventional method of chemical fixation, dehydration and embedding is the wrong stabilization method because the first step - the chemical fixation - already causes severe artifacts: the change of physicochemical properties of cellular proteins, hence water and ion redistribution and structure changes are inevitable. In recent years it became evident that only properly used cryotechniques are suited to stabilize living cells in such a way that the life-like localization of all cellular components including mobile ions and water are possible (see e.g., Steinbrecht and Zierold, 1987). In the following sections cryo-experiments with the striated muscle of the frog are discussed which already have been completed and experiments which should be performed in the future to obtain information on the physical state of ions and water in a representative living cell.

## The Subcellular Distribution of Mobile Ions in the Striated Muscle

MPT and AIH predict different subcellular distribution patterns of $K^+$ in the striated muscle according to the following considerations. The well known I band and A band areas of the muscle cell differ in their protein and water contents. Since the water content is higher in the I band compared to the A band (Huxley and Niedergerke, 1958) the MPT predicts that the freely dissolved cations are preferentially localized in the I band. The AIH predicts a preferential accumulation in the A band because $\beta$- and $\gamma$-carboxyl groups - the postulated sites of $K^+$ adsorption - are primarily found on myosin in the A band (Ling and Ochsenfeld, 1966). A test of these opposing predictions is also feasible with $Na^+$, $Rb^+$, $Cs^+$ and $Tl^+$. In frog muscle $K^+$ can be replaced reversibly in a mole-for-mole fashion with these different ions (Ling and Bohr, 1971; Ling, 1977b). If $Na^+$, $Rb^+$, $Cs^+$ or $Tl^+$ is the main cellular cation instead of $K^+$ the subcellular distribution of the respective ion should be the same as that of $K^+$ in a normal $K^+$ containing muscle.

Different properties of these different ions allow

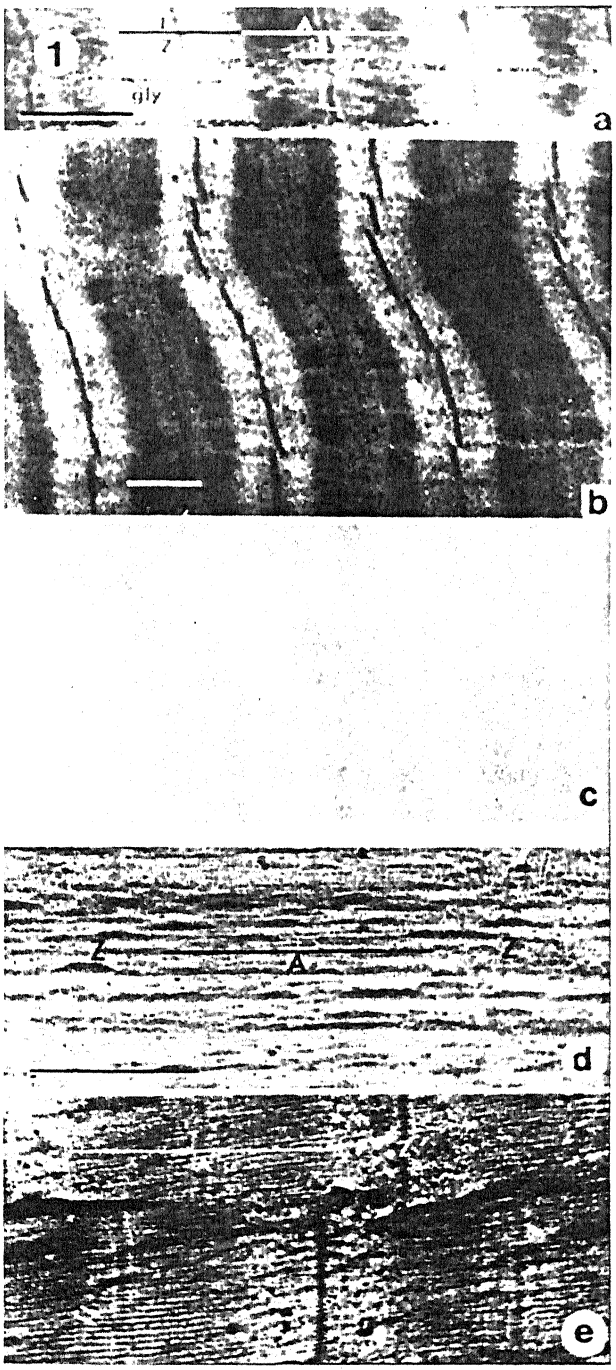


Figure 1: Subcellular distribution of $Cs^+$ and $Tl^+$ in frog sartorius muscle. Living muscles had been loaded with $Cs^+$ (a) and $Tl^+$ (b, e) prior to cryofixation. (a-c) Dry cut sections of freeze-dried and embedded preparations (freeze-drying equipment described by Edelmann, 1978; procedure: freeze-drying for 3 days at -80°C and for additional 6 days at -60°C, breaking the vacuum by dry nitrogen gas, infiltration in Spurr's resin at about -15°C for about 15h, polymerization at 40-50°C. (a) $Cs^+$-loaded muscle. (b) $Tl^+$-loaded muscle. (c) Control for (a) and (b): normal $K^+$ containing muscle. The dark areas in (a) and (b) represent the protein sites which preferentially adsorb $Cs^+$ (a) and $Tl^+$ (b). Abbreviations: **A**, A band; **H**, H zone; **M**, M line; **L**, L zone; **I**, I band; **Z**, Z line; gly, glycogen. (d, e) Frozen-hydrated cryosections. (d) Normal $K^+$ containing muscle. Only very faint ultrastructural details are visible. (e) $Tl^+$ containing muscle. Dark myosin filaments (arrows) in the A bands (A) and dark Z lines (Z) indicate preferential $Tl^+$ accumulation in the living cell. Bars: 1 $\mu$m. (a) from Edelmann (1977); (b, c) from Edelmann (1984a); (d,e) from Edelmann (1988). Reprinted by permission.

---

different methods of ion localization. An uneven distribution of $Rb^+$, $Cs^+$ or $Tl^+$ in a thin muscle section can be visualized directly under the electron microscope because these ions with high atomic numbers cause improved image contrast. $Na^+$, $Rb^+$, $Cs^+$ can be labeled with radioactive isotopes which are well suited for autoradiographic studies.

Ion localization was investigated by the following independent methods:

(1) Autoradiography of air dried single fibers using $^{134}Cs$ and $^{205}Tl$ (Ling, 1977a).

(2) Analysis of sections of freeze-dried, embedded (Spurr medium) muscle, (a) Visualization of $K^+$, $Rb^+$, $Cs^+$, $Tl^+$ by transmission electron microscopy (TEM) (Edelmann, 1977: $Cs^+$, $Tl^+$; Edelmann, 1980a: $K^+$, $Rb^+$, $Cs^+$; Edelmann, 1984a: $Cs^+$, $Tl^+$), (b) Electron probe X-ray microanalysis (EPXMA) of $K^+$, $Cs^+$, $Tl^+$ (Edelmann, 1978).

(3) Autoradiography of frozen hydrated single fibers at -196°C with $^{86}Rb$, $^{134}Cs$, and $^{22}Na$ (Edelmann, 1980a: $Rb^+$, $Cs^+$; Edelmann, 1986a: $Na^+$).

(4) EPXMA of $K^+$ in air-dried myofibrils (Trombitas and Tigyi-Sebes, 1979).

(5) EPXMA of $K^+$, $Rb^+$, $Cs^+$, $Tl^+$ in freeze-dried cryosections (Edelmann, 1983).

(6) Visualization of $Tl^+$ by TEM in frozen hydrated cryosections (Edelmann, 1984b, 1988, 1991a).

(7) EPXMA of $K^+$ in freeze-dried and Lowicryl K11M embedded muscle (Edelmann, 1986b).

(8) Visualization of $Cs^+$, $Tl^+$ by TEM in freeze-substituted and Lowicryl K11M embedded muscle

(Edelmann, 1988).

(9) EPXMA of $Na^+$ in freeze-dried cryosections (Edelmann, 1988).

(10) EPXMA of $K^+$, $Na^+$ in freeze-dried cryosections (von Zglinicki, 1988).

These independent methods yielded the following consistent results: the main cellular cation of a resting muscle cell, whether it is $Na^+$, $K^+$, $Rb^+$, $Cs^+$ or $Tl^+$ is preferentially found in the A band, especially at the outer edges and at the Z lines (see Fig. 1). Most important are the results obtained with frozen-hydrated preparations (Fig. 1e) as they confirm results obtained after freeze-drying (FD) or even freeze-substitution (FS). Although most results are qualitative, von Zglinicki (1988) has provided quantitative data which are in agreement with the qualitative observations. From these findings it is concluded:

(1) The results confirm predictions of the AIH,

(2) The concept that a living cell is a bag of ions freely dissolved in cell-water (MPT) is not tenable (Gupta, 1991, p. 42),

(3) The results obtained with ion sensitive microelectrodes and frog muscle cells must be an artifact (Ling *et al.*, 1973; Ling, 1984, pp 250-256; see also detailed discussion by Edelmann, 1989b)

(4) Cellular water must be maintained in a higher state of order than normal free water. This follows from the experiments of Hill and Kupalov (1930, cited above) but following a reverse direction of the logic of Hill and Kupalov: since the cellular water has the same activity as the extracellular water and the cellular cations are not free the reduction of water activity to that of a 0.1 M NaCl solution must be due to some kind of ordering of the cell water by cellular macromolecules.

One may argue that the observed protein-dependent subcellular ion distribution does not prove selective adsorption of different alkali-metal ions at the negative sites of cellular proteins (reviewed by Gupta, 1991, p. 42). However, the EMOC experiments cited above (Ling, 1978) show that the selective uptake of $K^+$ is caused by sites of cytoplasmic proteins and not by pumps.

In addition, it has been demonstrated *in vitro* that protein sites of freeze-dried and embedded muscle are able to adsorb selectively alkali-metal ions. 0.2 $\mu$m thick sections have been exposed to aqueous solutions containing various combinations of the salts LiCl, NaCl, KCl, and CsCl. EPXMA and laser microprobe mass analysis (LAMMA) of these sections show selective and preferential accumulation of $K^+$ (and $Cs^+$) over $Na^+$ on specific protein sites in the A bands; the total amount of adsorbed alkali-metal ions is in the same order of magnitude as the amount of accumulated cations in living frog muscle (Edelmann, 1980b, 1981). When using a LiCl-CsCl solution the section is heavily stained by the uptake of the electron-dense $Cs^+$ at the same sites which accumulate this ion in living muscle (Edelmann, 1984a); the adsorbing-capacity is lost in glutaraldehyde fixed muscle (Edelmann, 1986a, Fig. 2, see also ion-adsorption studies with freeze-substituted and Lowicryl embedded muscle, Edelmann, 1988, 1991b, and discussion by Ling, 1992a, b).

These experiments show that proteins maintained in its living-like state (through cryofixation and FD) behave completely different than isolated proteins (Lewis and Saroff, 1957) or proteins of cells killed e.g., by glutaraldehyde. Taken together, the observed selective accumulation of alkali-metal ions at specific protein sites of muscle cells - the ions being accumulated either in living cells or after FD and embedding - does not require membrane-situated pumps for its explanation.

The reviewed results leave many open questions most of which can only be answered by future microanalytical studies. Quantitative data of the following different preparations should be provided.

(1) Quantitative data of the subcellular distribution of electron-dense ions in ultrathin frozen-hydrated cryosections evaluated by microdensitometry. These data may then be used as a control for the optimal development of other techniques such as FD of cryosections and FD or FS of bulk specimens followed by embedding and subsequent microanalysis. The observation that different artifactual ion distribution patterns may result from different FD procedures (see Edelmann, 1994) and from different FS procedures clearly demand a precise control by an independent superior technique.

(2) Quantitative data of the subcellular ion distribution (e.g., $Na^+$, $K^+$, $Rb^+$, $Cs^+$) in EMOC preparations. A microanalytical study may directly establish the selective uptake of ions by cellular protein sites.

(3) Quantitative data of the uptake of different alkali-metal ions at different adsorption sites after exposure of frog muscle to different salt solutions. It has been shown that $Cl^-$ salts of $Li^+$, $Na^+$, $K^+$, $Rb^+$, $Cs^+$ cause different degrees of muscle swelling (Ling and Peterson, 1977). This finding has been interpreted by assuming that the different ions cause different degrees of dissociation of salt linkages between cellular proteins thus giving rise to different amounts of ion adsorption sites (Ling, 1984, p. 447 ff). This interpretation may be tested by cryopreparation and subsequent microanalysis.

(4) Quantitative data on the postulated redistri-

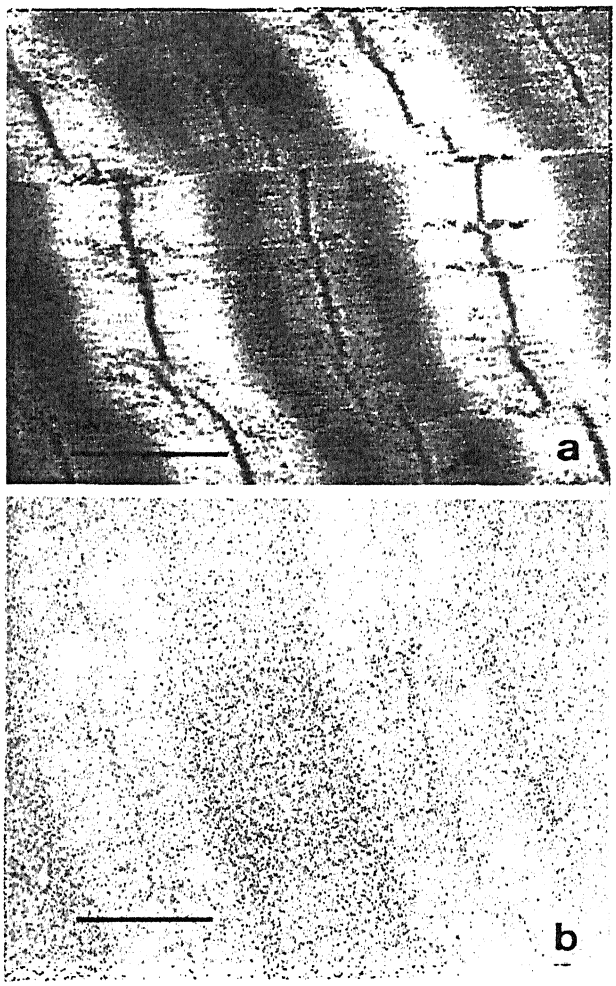


Figure 2: 0.2$\mu$m thick sections of frog sartorius muscle stained with a solution containing 100mM LiCl and 10mM CsCl. (For details of the method see Edelmann, 1984a, 1991). (a) Freeze-dried embedded muscle. (b) Glutaraldehyde fixed and embedded muscle. Bars: 1 $\mu$m. $Cs^+$ binding sites are only visualized in the freeze-dried preparation (from Edelmann, 1986a, reprinted by permission).

---

bution of $K^+$ during muscle contraction. The problem of muscle contraction is far from being solved (see Oplatka, 1996). Strangely enough, in today's accepted models of muscle contraction cell water plays no specific role. However, as Ling (1984, p. 566) pointed out shortening of a contracting living muscle is primarily an event of water movement. In a tentative model of muscle contraction based on principles of the AIH, Ling proposed that local liberation of adsorbed $K^+$ and local differences of water activities may be the cause of water shifts within sarcomeres of the contracting living muscle. Preliminary EM studies have already shown redistribution of $Cs^+$ or $Tl^+$ during tetanic contraction of $Cs^+$- or $Tl^+$-loaded muscles (Edelmann, 1989a). A detailed microanalytic study of the redistribution of $K^+$ during contraction of a living $K^+$ containing muscle would probably be the most important basis for a real understanding of this physiological process. The following theoretical concept (Ling and Ochsenfeld, 1991) derived from available data from the literature may be tested. In the resting muscle $\beta$- and $\gamma$-carboxyl groups of myosin heads are occupied by $K^+$. These anionic groups and cationic groups on actin form cross-bridges during muscle contraction. "When the fixed cationic site on an actin molecule successfully combines with the (actin-binding) $\beta$- or $\gamma$-carboxyl groups on a myosin head, a salt-linkage is formed. Salt-linkage formation of this kind would then represent the force-generating, cross-bridge-formation step of contraction. The subsequent displacement by $K^+$ of the $\epsilon$-amino or guanidyl groups on actin from the $\beta$- and $\gamma$-carboxyl groups on the myosin head then represents the relaxing, cross-bridge-dissociation step of a contraction cycle" (Ling and Ochsenfeld, 1991, p. 158).

## Cellular Water: a Challenging Problem for Cryotechniques

In the foregoing section it has been shown that the detected uneven distribution of the main cellular cation in the striated muscle leads to the conclusion that the molecule-to-molecule interaction in the bulk phase water must be different from normal free water. The question arises whether results obtained with cryo-experiments can be or have been explained by the different behavior of free and more ordered water. At first sight not much information from published experiments is available although it is evident that most if not all of the observed artifacts introduced by the cryotechniques is related to the water problem (see e.g., Kellenberger, 1987; Steinbrecht and Müller, 1987). However, the focus of interest was in general an optimized structure preservation it was not the water itself. The main reason for not looking at the properties of different water structures is probably the generally adopted conviction that the bulk of cell water is not differently structured than normal free water. But the following experiments clearly show that biological and non-biological preparations can be made which contain differently structured water. It has been shown that differently structured water can be identified by looking at its solute-exclusion property. Aqueous solutions of the "*extroverts*" gelatin, denatured hemoglobin, polyvinylpyrrolidone (PVP), polyethylene oxide (PEO), polyvinyl methylether

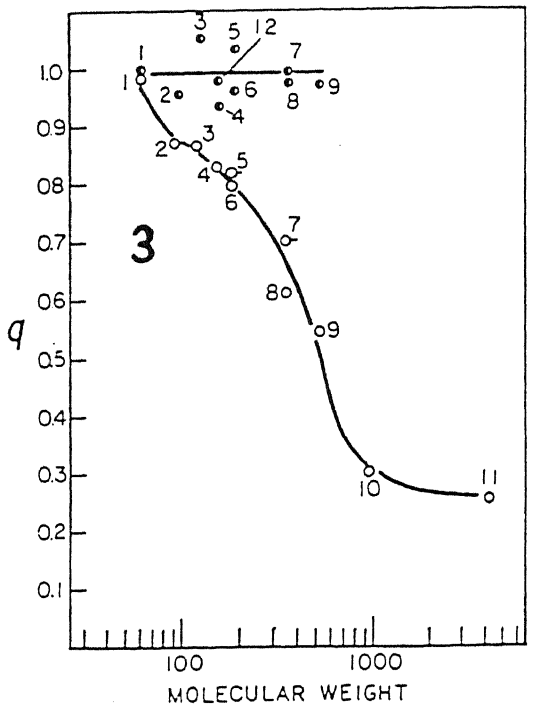


Figure 3: The equilibrium distribution coefficients (q-values) of various solutes in solutions of alkali-denatured hemoglobin (19%) at alkaline pH (empty circles) and of native hemoglobin (39%) at neutral pH (half-filled circles) plotted against their respective molecular weights. $q = [s]_{in}/[s]_{ex}$ where $[s]_{in}$ and $[s]_{ex}$ are the equilibrium solute concentration in the protein solution held in a dialysis sac and in the external bathing solution respectively 1, ethylene glycol; 2, glycerol; 3, erythritol; 4, xylitol; 5, sorbitol; 6, D-mannitol; 7, sucrose; 8, trehalose; 9, raffinose; 10, inulin; 11, PEG-4000, 12, D-xylose. Note that the solutes are not excluded ($q \approx 1$) from the water of the solution of native hemoglobin (*introvert*), but they are excluded from the water of alkali-denaturated hemoglobin (*extrovert*) in a size-dependent manner (from Ling and Hu, 1988; reprinted by permission).

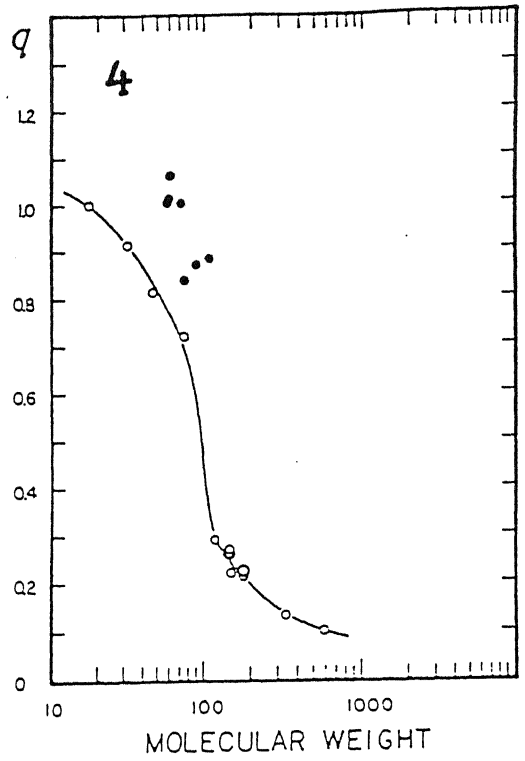


Figure 4: The equilibrium distribution coefficients (q-values) of twenty-one nonelectrolytes in frog muscle cells plotted against their respective molecular weights. $q = [s]_{in}/[s]_{ex}$ where $[s]_{in}$ represents the equilibrium solute concentration in cell water and $[s]_{ex}$ the equilibrium solute concentration of the external solution. The nonelectrolytes and their molecular weights and experimentally determined q-values are: methanol (32.04, 0.91); ethanol (46.07, 0.81); acetamide* (59.06, 1.00); urea* (60.10, 1.05); ethylene glycol* (62.07, 1.02); 1,2 propanediol* (76.10, 0.834); dimethylsulfoxide DMSO (78.14, 0.72); 1,2 butanediol* (90.12, 0.87); glycerol* (92.09, 1.00); 3-chloro-1,2 propanediol* (110.5, 0.893); erythritol (122.1, 029); D-arabinose (150.1, 0.27); L-arabinose (150.1, 0.27); L-xylose (150.1, 0.26); D-ribose (150.1, 0.26); xylitol (152.2, 0.22); D-glucose (180.2, 0.227); D-sorbitol (182.2, 0.227); D-mannitol (182.2, 0.217); sucrose (342.3, 0.132): D-raffinose (594.5, 0.100). Note the similarity of solute exclusion from living cells and from the *extrovert* model shown in Fig. 3. Note also the "aberrant" solutes (solid circles) of this figure (marked with *). Most of these solutes are known as cryoprotectants and it is assumed that they fit better than most other solutes into the dynamic water structure. From Ling *et al.* (1993), reprinted by permission.

---

(PVME) exhibit a reduced solvency (hence a partial exclusion) for electrolytes like $Na^+$ (Ling *et al.*, 1980) and for non-electrolytes like sugars (Fig. 3, Ling and Hu, 1988). In contrast, aqueous solutions of most native proteins (including hemoglobin, see Fig. 3) do not exhibit reduced solvency (so-called "*introverts*"). The reduction of solvency of the *extroverts* is size dependent i.e., the larger the solute, the lower the solvency (for reviews see Ling, 1992a,b). The partial exclusion of solutes from cellular water of living frog

muscle cells is size dependent similarly as found in *extrovert* preparations (Fig. 4, Ling *et al.*, 1993). These solutes are not excluded from cellular water of killed (e.g., by glutaraldehyde fixation) muscle cells.

More recently, Ling (1993) has derived a quantitative theory of solute distribution in water which is polarized in multilayers. The theory has made it possible for the first time to estimate quantitatively the intensities of (bulk phase) water polarization and to understand the action of cryoprotectants on cell water (enhanced stability of the polarized-multilayer state, see Ling *et al.*, 1993). The phenomena described above agree in general with the predictions of this new theory. With this information one may design systematic cryoexperiments with preparations containing differently structured water. Similar experiments should be carried out with polymer solutions, with solutions of proteins, e.g., of NaOH denatured hemoglobin (*extrovert*) and of native hemoglobin (*introvert*), and with living and killed muscle. The muscle preparation is of particular interest because its water content can be manipulated without changing the content of other cellular compounds: Ling and Negendank (1970) have shown that the water uptake of frog muscle cells exposed to different concentrations of water vapor obeys Bradley's polarized multilayer adsorption isotherm of water vapor adsorption (already mentioned above). By using the water vapor equilibration technique the cellular water may be reduced by removing more and more water layers which are less polarized than the first layers surrounding the water influencing protein. Hence, the remaining water layers should eventually exhibit an optimally enhanced structure.

In the following, cryo-experiments are discussed which may be suited to investigate differently structured water.

## Rapid Freezing and Vitrification

With the development of cryofixation techniques and of cryo electron microscopy water became a suitable substance for electron microscopy (Dubochet *et al.*, 1987, 1988). It turned out that water in biological material or model systems can be vitrified which means that the water is solidified by rapid freezing without allowing it to crystallize before cryofixation is completed. Frozen hydrated specimens (e.g., frozen liquid films or cryosections) can be examined in the cryo electron microscope and the depth of vitrification of a cryofixed preparation can be evaluated by electron diffraction. It is assumed that structural and redistribution artifacts are minimized in the vitrified area. Examination of biological specimens cryofixed at ambient pressure showed that extracellular fluid (as well as pure water) cannot be vitrified over more than about 1 $\mu$m whereas uncryoprotected tissue (e.g., liver, kidney) can be vitrified up to a depth of 5-10 $\mu$m (Dubochet *et al.*, 1987). It is assumed that this latter value is possible because the cell interior contains a high concentration of soluble material which plays the role of a cryoprotectant. However this interpretation need not be correct. With the concept of the AIH that most of intracellular mobile compounds are adsorbed to macromolecules and that the water of living cells is differently structured than extracellular water one may expect a different freezing behavior of the different water structures. Slow freezing experiments have already shown that the freezing of *extrovert* and *introvert* solutions is completely different (Luyet and Rapatz, 1956, discussed by Ling, 1992a): solutions of *introverts* at low and high concentrations show regular hexagonal ice formation, whereas irregular dendritic rosettes are formed in *extrovert* solutions.

Microcalorimetric studies have also shown that slow freezing of *introverts* and *extroverts* is completely different. Solutions of *introverts* at low and high concentrations exhibit unchanging freezing temperature and rates of freezing whereas freezing rate and temperature decreases with increasing concentration of the macromolecule investigated (Ling and Zhang, 1983). For instance, a 50 % native bovine hemoglobin solution freezes the same way as a 5 % solutions whereas a 50 % PVME solution solidifies without ice crystal formation. One may therefore expect that the depth of vitrification of *extrovert* solutions after rapid cooling is increasing with increasing concentration and that the water of *introvert* solutions freezes more or less like pure water. This expectation may be tested with the different model systems as well as with living and dead cells by determining the depth of vitrification after standardized cryofixation.

From the determination of the devitrification temperature DT (temperature at which the vitrified water is converted to cubic ice) of different water specimens one expects additional information on the different kinds of water: it is known that the DT of pure water is in the range of about -125°C but devitrification starts already at about -138°C (reviewed by Bachmann and Mayer, 1987). From the finding that solutions with high concentrations of *extroverts* solidify at low temperature without ice crystal growth one may expect that the DT of a specimen is highly dependent on the degree of organization of its water. It must be noted here that the devitrification process cannot be observed directly in the cryo electron microscope if the DT is rather high. The water will

sublime before the transition from vitreous to crystalline ice takes place. Therefore one has to keep the cryofixed material for different periods at atmospheric pressure at the supposed DT, to cool the specimen again and to observe electron diffraction in the cryo electron microscope.

If it turns out that the DT is dependent on the water structure of the investigated system one may try to relate the DT to the amount of surface modified water and its degree of polarization. This relationship may then be used to investigate the water of living cells and even of subcellular areas. Another problem may be tackled: during specimen preparation and extreme events such as application of high pressure to the biological specimen before freezing by the high pressure freezing technique changes in protein and water structure may occur before the specimen is solidified (for artifacts occurring during HPF see Ding *et al.*, 1992; Wallén and Hallberg, 1993; Leforestier *et al.*, 1996). Determination of the DT may be used to study the degree of water structure change during the preparative steps. Finally, a consequence for practical work should be mentioned: investigation of the DT may help to answer the question whether certain cryofixed biological specimens (not cryoprotected) can be cryosectioned at a rather high subzero temperature without artifacts which are normally observed in cryosections containing cubic or hexagonal ice (Saubermann, 1981; for discussion and review of this problem see Sitte, 1996). One may assume that vitrified more ordered water systems are stable at higher subzero temperatures than vitrified pure water.

It would be of course best to detect directly differences of water structure in vitrified samples by electron diffraction. The problem is certainly not easy because of the more or less random distribution of molecules in the *extrovert* preparation and the highly dynamic nature of the water structure in solutions and living cells (Ling, 1992a). However, one may try to detect water layers in muscles with enhanced water structure as discussed above or to look for even better suited water ordering structures.

## Dehydration of Cryofixed Specimens by Freeze-Drying

It was claimed in 1986 that a special procedure of freeze-drying (FD) can remove amorphous phase tissue water without devitrification making possible ultrastructural localization of soluble molecular entities without the problem of alteration, redistribution, and loss (Linner *et al.*, 1986). Unfortunately this claim was not correct (discussed by Edelmann, 1994; Sitte *et al.*, 1994). So far FD has not been performed without changing the ultrastructure seen in frozen-hydrated preparations or without shrinkage artifacts. This is not surprising because specific structures of macromolecules can only exist by interaction with water molecules which therefore cannot be replaced by other substances or the vacuum without artifact formation (Bachmann and Mayer, 1987; Kellenberger, 1987). Taking into account that during freeze-substitution (FS) the water molecules with a dipole moment of 1.86 Debye are usually replaced with dipolar molecules (acetone: 2.89 Debye, methanol: 1.70 Debye) it can be understood why so far less morphological changes are obtained after FS than after FD. However, FD can be studied directly by electron microscopy and the question arises whether one may obtain information on the degree of interaction between biological macromolecules and the surrounding water by FD studies. According to the general view a mono- or bilayer of water is surface-modified and behaves substantially different from bulk water (Bachmann and Mayer, 1987). According to the AIH almost all the water of living cells is surface-modified as established with frog muscle cells by analysis of the size-dependent solvency of different solutes (Ling *et al.*, 1993). Taking into account that the water of *extrovert* solutions is polarized in multilayers similarly as muscle cell water (the intensity of polarization is however lower in the *extrovert* solutions) and that most of the water of *introvert* solutions behaves like free water one may perform FD studies aimed to answer the following questions. Is the sublimation rate at a given temperature dependent on the polarization of the investigated water? Is the structure preservation of the macromolecules after FD dependent on their efficiency to polarize water? Is the sublimation rate different in the areas of differently solidified water (vitreous, cubic, hexagonal)?

If these questions can be answered positively one may derive a method by means of which it should be possible to judge the interactions between proteins and water in living and dead cells and to derive optimal FD procedures for biological material.

An indication for a positive answer to the first question can already be found in FD experiments of biological material (Edelmann and Ruf, 1996): Wet-cut ultrathin sections of freeze-dried and Lowicryl HM20 embedded human lymphocytes (not treated with chemical fixatives) are distorted in the areas of best cryofixed cells (Fig. 5a, compare with Fig 5b, which shows a section from the same Lowicryl block, after stabilization with $OsO_4$ vapor). It is assumed that in the region of best cryofixation (vitrification) a relative strong protein-water interaction within the cells is preserved leading to a reduced rate of water removal

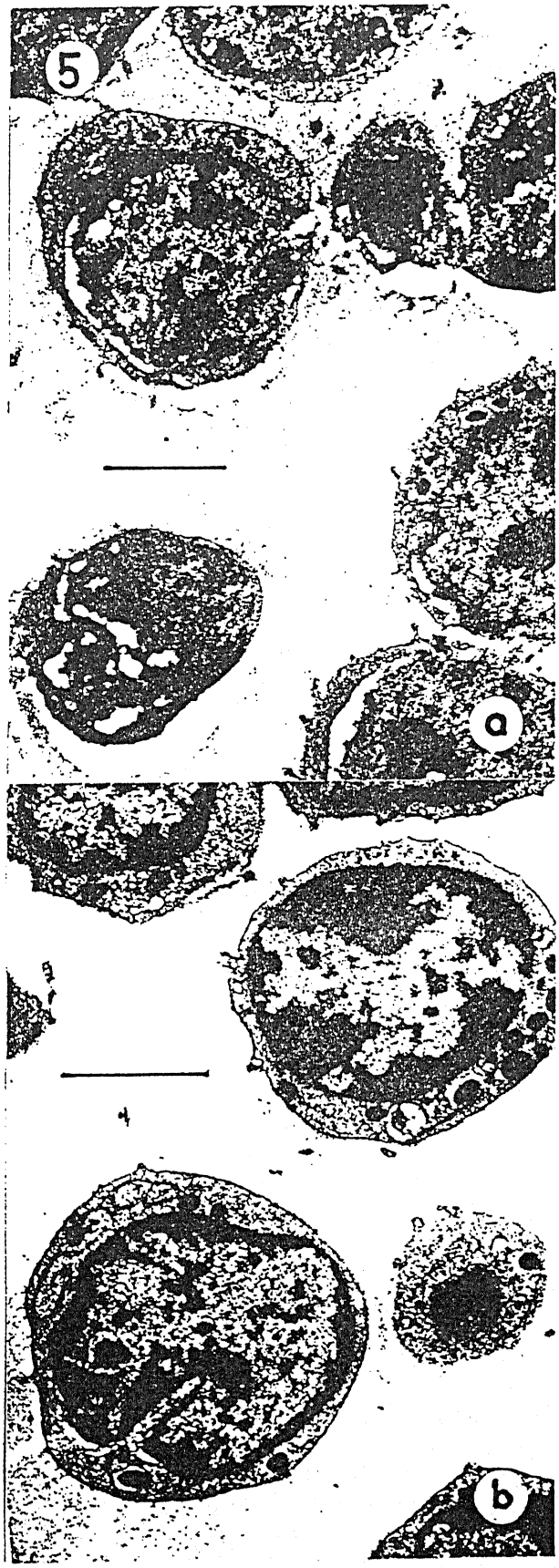


Figure 5: Ultrathin sections of human lymphocytes after freeze drying FD and low temperature embedding in Lowicryl HM20 at -25°C. FD: 1 day -100°C, temperature increase 0.9°C/h up to -10°C, 1 day -10°C. Uranyl acetate and lead citrate staining. (a) Compression and extraction artifacts occurring during wet-cutting are pronounced near the freezing plane (left lower corner). For interpretation of these artifacts see text. (b) This section was obtained from the same Lowicryl block but after exposure of the block for 30 min to $OsO_4$ vapor. Apparently the whole resin-biological material-complex is stabilized and the ultrastructure is well preserved. Freezing plane: left lower corner. Scale bars: 2 $\mu$m (from Edelmann and Ruf, 1996, reprinted by permission).

---

during FD and hence to less dry areas of the biological material. If more water molecules are retained in certain areas polymerization of the Lowicryl may be incomplete and lead to the observed distortion during wet-cutting. Whether this interpretation is correct may be tested by experiments with cryofixed *extroverts* and *introverts*. It is expected that after similar FD of both model systems less water is retained in *introverts* than in *extroverts*.

## Conclusion

Understanding basic cellular functions requires a detailed knowledge of the intracellular interactions between the main cellular components water, proteins, and $K^+$ ions. These interactions are not directly accessible to current biochemical methods but it is now possible to test existing theories of these interactions by investigating the striated muscle and suitable model systems by means of cryotechniques and analytical electron microscopy.

## Acknowledgement

I would like to thank Mrs. B Reiland for typing the manuscript.

We do not encourage references to entire books!

## References

Bachmann L, Mayer E (1987). Physics of water and ice: Implications for cryofixation. In: Cryotechniques in Biological Electron Microscopy. Steinbrecht RA, Zierold K, (eds). Springer-Verlag, Berlin-Heidelberg, pp 3-34.

Bradley RS (1936). Polymolecular adsorbed films. II. The general theory of the condensation of vapors in finely divided solids. J Chem Soc 1936: 1799-1804.

Carr CW (1956). Studies on the binding of small ions in protein solutions with the use of membrane electrodes. VI. The binding of sodium and potassium ions in solutions of various proteins. Arch Biochem Biophys 62: 476-484.

Ding, B, Turgeon R, Parthasarathy MV (1992). Effect of high pressure freezing on plant microfilament bundles. J Microsc 165: 367-376.

Dubochet J, Adrian M, Chang JJ, Lepault J, McDowall AW (1987). Cryo-electron microscopy of vitrified specimens. In: Cryotechniques in Biological Electron Microscopy. Steinbrecht RA, Zierold K (eds). Springer-Verlag, Berlin-Heidelberg, pp 112-131.

Dubochet J, Adrian M, Chang JJ, Homo JC, Lepault J, McDowall AW, Schultz P (1988). Cryo-electron microscopy of vitrified specimens. Quart Rev Biophys 21: 129-228.

Edelmann L (1973). The influence of rubidium and cesium ions on the resting potential of guinea-pig heart muscle cells as predicted by the association-induction hypothesis. Ann NY Acad Sci 204: 534-537.

Edelmann L (1977). Potassium adsorption sites in frog muscle visualized by cesium and thallium under the transmission electron microscope. Physiol Chem Phys 9: 313-317.

Edelmann L (1978). Visualization and X-ray microanalysis of potassium tracers in freeze-dried and plastic embedded frog muscle. Microsc Acta Suppl 2: 166-174.

Edelmann L (1980a) Potassium binding sites in muscle. Electron microscopic visualization of K, Rb, and Cs in freeze-dried preparations and autoradiography at liquid nitrogen temperature using $^{86}Rb$ and $^{134}Cs$. Histochemistry 67: 233-242.

Edelmann L (1980b). Preferential localized uptake of $K^+$ and $Cs^+$ over $Na^+$ in the A band of freeze-dried embedded muscle section. Detection by X-ray microanalysis and laser microprobe analysis. Physiol Chem Phys 12: 509-514.

Edelmann L (1981). Selective accumulation of $Li^+$, $Na^+$, $K^+$, $Rb^+$, and $Cs^+$ at protein sites of freeze-dried embedded muscle detected by LAMMA. Fresenius Z Anal Chem 308: 218-220.

Edelmann L (1983). Electron probe X-ray microanalysis of K, Rb, Cs, and Tl in cryosections of the striated muscle. Physiol Chem Phys 15: 337-344.

Edelmann L (1984a). Subcellular distribution of potassium in striated muscles. Scanning Electron Microsc 1984; II: 875-888.

Edelmann L (1984b). Frozen hydrated cryosections of thallium loaded muscle reveal subcellular potassium binding sites. Physiol Chem Phys and Med NMR 16: 499-501.

Edelmann L (1986a). Two opposing theories of the cell: experimental testing by cryomethods and electron microscopy. In: The Science of Biological Specimen Preparation. Müller M, Becker RP, Boyde A, Wolosewick JJ, (eds). Scanning Electron Microscopy, Inc AMF O'Hare, Chicago, IL 60666, pp 33-42.

Edelmann L (1986b). Freeze-dried embedded specimens for biological microanalysis. Scanning Electron Microsc 1986; IV: 1337-1356.

Edelmann L (1988). The cell water problem posed by electron microscopic studies of ion binding in muscle. Scanning Microsc 2: 851-865.

Edelmann L (1989a). The contracting muscle: a challenge for freeze-substitution and low temperature embedding. Scanning Microsc Suppl 3: 241-252.

Edelmann L (1989b). The physical state of potassium in frog skeletal muscle studied by ion-sensitive microelectrodes and by electron microscopy: Interpretation of seemingly incompatible results. Scanning Microsc 3: 1219-1230.

Edelmann L (1991a). Freeze-substitution and the preservation of diffusible ions. J Microsc 161: 217-228.

Edelmann L (1991b). Adsorption staining of freeze-substituted and low temperature embedded frog skeletal muscle with cesium: a new method for the investigation of protein-ion interactions. Scanning Microsc Suppl 5: S75-S84.

Edelmann L (1994). Optimal freeze-drying of cryosections and bulk specimens for X-ray microanalysis. Scanning Microsc Suppl 8: 67-81.

Edelmann L, Ruf A (1996). Freeze-dried human leukocytes stabilized with uranyl acetate during low temperature embedding or with $OsO_4$ vapor after embedding. Scanning Microsc Suppl 10 (in press).

Ernst E (1963). Biophysics of the Striated Muscle, 2nd ed. Publishing House of Hungarian Academy of Sciences, Budapest. pp. 1 – 398.

Gersh I (1938). Improved histochemical methods for chloride, phosphate-carbonate and potassium applied to skeletal muscle. Anat Rec 70: 311-329.

Glynn JM, Karlish SJD (1975). The sodium pump. Ann Rev Physiol 37: 13-55.

Gupta BL (1991). Ted Hall and the science of biological microprobe X-ray analysis: a historical perspective of methodology and biological dividends. Scanning Microsc 5: 379-426.

Hill AV (1930). The state of water in muscle and blood and the osmotic behavior of muscle. Proc Royal Soc Ser B 106: 477-505.

Hill AV, Kupalov PS (1930). The vapor pressure of muscle. Proc Royal Soc Ser B 106: 445-475.

Hodgkin AL, Katz B (1949). The effect of sodium

on the electrical activity of the giant axon of the squid. J Physiol 108: 37-77.

Hoffmann EK, Dunham PhB (1995). Membrane mechanisms and intracellular signalling in cell volume regulation. Int Rev Cytol 161: 173-262.

Huang HW, Hunter SH, Warburton WK (1979). X-ray absorption edge fine structure of potassium ions in various environments: application to frog blood cells. Science 204: 191-193.

Huxley AF, Niedergerke R (1958). Measurement of the striations of isolated muscle fibers with the interference microscope. J Physiol 144: 403-425.

Jones AW (1965). The water and electrolytes metabolism of the arterial wall. Ph D Thesis Univ of Pennsylvania, Philadelphia. not yet found pages?

Kellenberger E (1987). The response of biological macromolecules and supra molecular structures to the physics of specimen preparation. In: Cryotechniques in Biological Electron Microscopy. Steinbrecht RA, Zierold K (eds). Springer-Verlag, Berlin-Heidelberg, pp 35-63.

Leforestier A, Richter K, Livolant F, Dubochet J (1996). Comparison of slam-freezing and high-pressure freezing effects on the DNA cholesteric liquid crystalline structure. J Microsc 184: 4-13.

Lewis MS Saroff HA (1957) The binding of ions to muscle proteins. Measurements on the binding of potassium and sodium ions to myosin A, myosin B and actin. J Am Chem Soc 79: 2112-2117.

Ling GN (1962). A Physical Theory of the Living State. The Association-Induction Hypothesis, Blaisdell Publ Co, Waltham, MA. pp. 1 - 630.

Ling GN (1965). The physical state of water in living cell and model systems. Ann NY Acad Sci 125: 401-417.

Ling GN (1977a). $K^+$ localization in muscle cells by autoradiography, and identification of $K^+$ adsorption sites in living muscle cells with uranium binding sites in electron micrographs of fixed cell preparation. Physiol Chem Phys 9: 319-328.

Ling GN (1977b). Thallium and cesium in muscle cells compete for the adsorption sites normally occupied by $K^+$. Physiol Chem Phys 9: 217-225.

Ling GN (1978). Maintenance of low sodium and high potassium levels in resting muscle cells. J Physiol (London) 280: 105-123.

Ling GN (1984). In Search of the Physical Basis of Life. Plenum Publishing Corp, New York, NY. pp. 1-791.

Ling GN (1990). Theory of active transport across frog skin and other bifacial cell systems. A subsidiary of the association-induction hypothesis. Scanning Microsc 4: 723-736.

Ling GN (1992a). A Revolution in the Physiology of the Living Cell. RA Krieger Publ Co, Melbourne, FL. pp. 1 - 378.

Ling GN (1992b). Can we see living structure in a cell? Scanning Microsc 6: 405-450.

Ling GN (1993). A quantitative theory of solute distribution in cell water according to molecular size. Physiol Chem Phys & Med NMR 25: 145-175.

Ling GN (1994). The new cell physiology: An outline, presented against its full historical background, beginning from the beginning. Physiol Chem Phys and Med NMR 26: 121-203.

Ling GN, Bohr G (1971). Studies on ion distribution in living cells: IV. Effects of ouabain on the equilibrium concentration of $Cs^+$, $Rb^+$, $K^+$, $Na^+$ and $Li^+$ ions in frog muscle cells. Physiol Chem Phys 3: 573-583.

Ling GN, Hu W (1988). Studies on the physical state of water in living cells and model systems. X. The dependence of the equilibrium distribution coefficient of a solute in polarized water on the molecular weights of the solute: experimental confirmation of the "size rule" in model studies. Physiol Chem Phys and Med NMR 20: 293-307.

Ling GN, Negendank W (1970). The physical state of water in frog muscles. Physiol Chem Phys 2: 15-33.

Ling GN, Negendank W (1980). Do isolated membranes and purified vesicles pump sodium? A critical review and reinterpretation. Persp Biol Med 23: 215-239.

Ling GN, Ochsenfeld MM (1966). Studies on ion accumulation in muscle cells. J Gen Physiol 49: 819-843.

Ling GN, Ochsenfeld MM (1991). The majority of potassium ions in muscle cells is adsorbed on the ß- and γ-carboxyl groups of myosin: potassium-ion-adsorbing carboxyl groups on myosin heads engage in cross-bridge formation during contraction. Physiol Chem Phys & Med NNM 23: 133-160.

Ling GN, Peterson K (1977). A theory of cell swelling in high concentrations of KCl and other chloride salts. Bull of Math Biol 39: 721-741.

Ling GN, Tucker M (1983). Only solid red blood cell ghosts transport $K^+$, and $Na^+$ against concentration gradients: hollow intact ghosts with $K^+$-$Na^+$ activated ATPase do not. Physiol Chem Phys and Med NMR 15: 311-317.

Ling GN, Walton CL (1976). What retains water in living cells? Science 191: 293-295.

Ling GN, Zhang ZL (1983). Studies on the physical state of water in living cells and model systems. IV. Freezing and thawing point depression of water gelatin, oxygen-containing polymers and urea-denatured proteins. Physiol Chem Phys & Med NMR 15: 391-406.

Ling GN, Miller C, Ochsenfeld MM (1973). The physical state of solutes and water in living cells according to the association-induction hypothesis. Ann NY Acad Sci 204: 6-50.

Ling GN, Ochsenfeld MM, Walton C, Bersinger TJ (1980). Mechanism of solute exclusion from cells: The role of protein-water interaction. Physiol Chem Phys 12: 3-10.

Ling GN, Niu Z, Ochsenfeld MM (1993). Predictions of polarized multilayer theory of solute distribution confirmed from a study of the equilibrium distribution in frog muscle of twenty-one nonelectrolytes including five cryoprotectants. Physiol Chem Phys & Med NMR 25: 177-208.

Linner JG, Livesey SA, Harrison DS, Steiner AL (1986). A new technique for removal of amorphous phase tissue water without ice crystal damage: A preparative method for ultrastructural analysis and immunoelectron microscopy. J Histochem Cytochem 34: 1113-1135.

Luyet B, Rapatz F (1956). Patterns of ice formation in some aqueous solutions. Biodynamica 8: 1-67.

Macallum AB (1905). On the distribution of potassium in animal and vegetable cells. J Physiol 32: 95-128.

Minkoff L, Damadian R (1973). Caloric catastrophe. Biophys J 13: 167-178.

Negendank W (1986). The state of water in the cell. In: The Science of Biological Specimen Preparation. Müller M, Becker RP, Boyde A, Wolosewick JJ (eds). Scanning Electron Microscopy Inc, AMF O'Hare Chicago, IL, 60666, pp 21-32.

Oplatka A (1996). The rise, decline, and fall of the swinging crossbridge dogma. Chemtracts - Biochem Molec Biol 6: 18-60.

Rorschach HE, Bearden DW, Hazlewood CF, Heidorn DB, Nicklow RM (1987). Quasi-elastic scattering studies of water diffusion. Scanning Microsc 1: 2043-2049.

Saubermann AJ (1981). Cryosectioning of biological tissue for X-ray microanalysis of diffusible elements. In: Microprobe Analysis of Biological Systems. Hutchinson TE, Somlyo AP (eds). Academic Press, New York and London, pp 377-396.

Sitte H (1996) Advanced instrumentation and methodology related to cryo-ultramicrotomy: a review. Scanning Microsc Suppl 10: (in press). we will fix

Sitte H, Edelmann L, Hässig H, Kleber H, Lang A (1994). A new versatile system for freeze-substitution, freeze-drying and low temperature embedding of biological specimens. Scanning Microsc Suppl 8: 47-66.

Steinbrecht RA, Müller M (1987). Freeze-substitution and freeze-drying. In: Cryotechniques in Biological Electron Microscopy. Steinbrecht RA, Zierold K (eds). Springer-Verlag, Berlin-Heidelberg, pp 149-172.

Steinbrecht RA, Zierold K (eds) (1987). Cryotechniques in biological electron microscopy. Springer-Verlag, Berlin-Heidelberg. pages? pp.1-297.

Trantham EC, Rorschach HE, Clegg JS, Hazlewood CF, Nicklow RM, Wakabayashi N (1984). Diffusive properties of water in Artemia cysts as determined from quasi-elastic neutron scattering spectra. Biophys J 45: 927-938.

Trombitas K, Tigyi-Sebes A (1979). X-ray microanalytical studies on native myofibril and mitochondria isolated by microdissection from honey-bee flight muscle. Acta Biochim Biophys Acad Sci Hung 14: 271-277.

Troshin AS (1958). Das Problem der Zellpermeabilitaet (The Problem of Cell Permeability). Gustav Fischer Verlag, Jena, Germany. pp.1-396. pages

Troshin AS (1966). The Problem of Cell Permeability. Pergamon Press, New York. pp.1-543. pages

Von Zglinicki T (1988). Monovalent ions are spatially bound within the sarcomere. Gen Physiol Biophys 7: 495-504.

Wallén R, Hallberg E (1993). Preservation of insect peripheral nervous tissues. In: Extended Abstracts of the 45th Annual Meeting of the Scandinavian Society for Electron Microscopy (Karlsson G, ed) BTJ-tryck AB, Lund, Sweden, pp 69-70.

## Discussion with Reviewers

J.S. Clegg: Whether those who believe the membrane-pump theory (MPT) - the overwhelming majority, as pointed out in the paper - will change their views, or even read the paper, seems to me to be very unlikely.

Reviewer IV: Most biologists find the evidence for membrane pumps so well established that the author's work would be a curiosity at best and considered ridiculous at worst. The paper is frankly of no significance and unlikely to be of general interest.

Author: I have expected such comments which are reflecting human behavior as expressed by Lavoisier (*Reflections on Phlogiston*): "The human mind gets creased into a way of seeing things. Those who have envisaged nature according to a certain point of view during much of their career, rise only with difficulty to new ideas." In my opinion, the basic concepts of the AIH will be accepted sooner or later. However, patience is required because the peer-review system favors conformity to the majority opinion and minority views are less likely to have research grant proposals

We cannot print your accusation of fraud! The way you have written it.

Ludwig, I have edited your reply. Our guidelines say that the answer should not be insulting and that complimentary phrases should be

funded or papers accepted by journals with high impact factors (see also Ling, 1997; Braben, 1996; and the discussion on "minority views" in Edelmann, 1988, p 865, text reference). Being aware of this situation I address this paper to those who are interested in new avenues of research.

C.F. Hazlewood: The discussion on the evidence for the organization of cell water in space and time should be expanded.

J.S. Clegg: The paper is essentially dealing with research that adopts the AIH. Citation of additional extensive studies of many scientists on the properties of cell water would make the paper stronger and more complete.

J.S. Clegg: I agree with the author that much, possibly all of the water in cells may differ in its properties (and therefore structure) from water in dilute aqueous solutions. However, I am not aware of direct and compelling experimental evidence that the structure must consist of polarized multilayers of water. Would you please respond to that?

Author: Many scientists have studied the properties of cell water with many different methods thereby confirming the view that certain properties of cellular water differ considerably from those of water in dilute aqueous solutions. Important results of different kinds of studies can be found in the following reviews or books (not a complete list): Hazlewood (1979); Ling (1984, text reference), Clegg (1984 a,b), Negendank (1986, text reference), Kasturi *et al.* (1987), Cameron *et al.* (1988 a,b), Rorschach *et al.* (1987, text reference), Clegg and Drost-Hansen (1991), Ling (1992a, text reference). I have emphasized the idea of polarization of cell water in multilayers (PM theory) which is in my opinion particularly attractive and compelling because of the following reasons: firstly, it is an integral part of the only self-consistent, unifying theory of cell physiology dealing with all major aspects of cell physiology - in contrast to the piecemeal, *ad hoc* MPT; secondly, the PM theory offers a physically sound theoretical mechanism for water under the influence of proteins assuming certain conformation, and it provides predictions which have been experimentally verified (what else can be expected from a scientific theory?): In agreement with the polarized multilayer theory of cell water, 95% of the water in frog muscle follows the Bradley polarized multilayer adsorption isotherm (Ling and Negendank, 1970, text reference). In agreement with theory water sorption on the *extrovert* gelatin also follows that isotherm (Ling, 1984, p. 288, text reference). In addition, by using statistical mechanics Ling (1993, text reference) developed a quantitative theory of solute distribution in water which is polarized in multilayers. From this theory three major predictions have been derived which are experimentally verified for water in *extrovert* solutions and for cell water (Ling *et al.*, 1993, text reference). These three predictions are: (1) *Linear distribution*: when the equilibrium concentration of a solute in cell (or model water) is plotted against its concentration in the external solution, a straight line is obtained, with a slope equal to the equilibrium distribution coefficient or q-value of that solution in cell water. (2) *The size-rule*: The q-value is, as a rule, size-dependent: the larger the solute, the lower the q-value. (3) *Solutes with exceptionally high q-value may act as cryoprotectants.*

G.M. Roomans: After death or (cardiac) infarct, the content of $K^+$ in (cardiac) muscle decreases, whereas $Na^+$ increases. This can easily be explained in the context of the MPT as being due to lack of ATP, and a decrease of the ATP content can be shown. In the AIH, a different explanation must be found (changes in protein conformation?). Are experimental data available to support such an alternative hypothesis?

Author: In the AIH, ATP serves its critical role not by its hydrolytic delivery of a package of energy stored in a special phosphate bond (a disproven idea, reviewed by Ling, 1992a, p.15, text reference). Rather ATP serves its function by virtue of its extremely strong interaction with the appropriate cardinal site, a theoretical postulation now established beyond any doubt by the accurate determination of the enormous binding constant of ATP (not shared by ADP) on myosin (see Ling, 1992a, p. 180). In theory, ATP adsorption on this site transforms a gang of cooperatively linked $\beta$- and $\gamma$-carboxyl groups into a $K^+$-preferring state (conformational change). Therefore a quantitative relationship between the equilibrium level of ATP in the cell and the equilibrium level of $K^+$ in the cell should exist regardless of how one alters the level of ATP in the cell (Ling, 1992a, p. 188, text reference). Gulati *et al.* (1971) studied the equilibrium concentration of ATP and of $K^+$ in frog muscles after exposure to 11 poisons of widely diverse toxicological effects. Their data show that the predicted linear relationship between ATP concentration and $K^+$ concentration in the cell does indeed hold. In addition, according to theory, ATP is responsible for water polarization in the cell. It has been shown that as ATP concentration fell in a specific frog muscle experiment where 0.2 mM iodoacetate was used, the changes of $K^+$ and $Na^+$ concentration are like mirror images as expected ($K^+$ down, $Na^+$ up). In this experiment a parallel

funded or papers accepted by journals with high impact factors (see also Ling, 1997; Braben, 1996; and the discussion on "minority views" in Edelmann, 1988, p 865, text reference). Being aware of this situation I address this paper to those who are interested in new avenues of research.

,Edelmann, 1982;

Replace by ⊗:

As a consequence most scientists are forced to adapt themselves to this policy and to avoid any contact with a new "heretical" idea (see Ling, 1997, linked page: lp12a.htm). Being aware of this situation I address this paper to those who are not completely dependent on our peer-review system and who are interested in new avenues of research.

C.F. Hazlewood: The dis
the organization of cell wa
be expanded.

J.S. Clegg: The paper
research that adopts the
extensive studies of many
of cell water would make
complete.

J.S. Clegg: I agree w
possibly all of the wate
properties (and therefore
dilute aqueous solutions.
direct and compelling ex
structure must consist
water. Would you please

Author: Many scientists
cell water with many
confirming the view that
water differ considerably
aqueous solutions. Impor
of studies can be found
books (not a complete li
(1984, text reference), (
(1986, text reference), K
*et al.* (1987), Cameron
*et al.* (1988 a,b), Rorschach *et al.* (1987, text reference), Clegg and Drost-Hansen (1991), Ling (1992a, text reference). I have emphasized the idea of polarization of cell water in multilayers (PM theory) which is in my opinion particularly attractive and compelling because of the following reasons: firstly, it is an integral part of the only self-consistent, unifying theory of cell physiology dealing with all major aspects of cell physiology - in contrast to the piecemeal, *ad hoc* MPT; secondly, the PM theory offers a physically sound theoretical mechanism for water under the influence of proteins assuming certain conformation, and it provides predictions which have been experimentally verified (what else can be expected from a scientific theory?): In agreement with the polarized multilayer theory of cell water, 95% of the water in frog muscle follows the Bradley polarized multilayer adsorption isotherm (Ling and Negendank, 1970, text reference). In agreement with theory water sorption on the *extrovert* gelatin also follows that isotherm (Ling, 1984, p. 288, text reference). In addition, by using statistical mechanics Ling (1993, text reference) developed a quantitative theory of solute distribution in water which is polarized in multilayers. From this theory three major predictions have been derived which are experimentally verified for water in *extrovert* solutions and for cell water (Ling *et al.*, 1993, text reference). These three predictions are: (1) *Linear distribution*: when the equilibrium concentration of a solute in cell (or model

cardinal site, a theoretical postulation now established beyond any doubt by the accurate determination of the enormous binding constant of ATP (not shared by ADP) on myosin (see Ling, 1992a, p. 180). In theory, ATP adsorption on this site transforms a gang of cooperatively linked $\beta$- and $\gamma$-carboxyl groups into a $K^+$-preferring state (conformational change). Therefore a quantitative relationship between the equilibrium level of ATP in the cell and the equilibrium level of $K^+$ in the cell should exist regardless of how one alters the level of ATP in the cell (Ling, 1992a, p. 188, text reference). Gulati *et al.* (1971) studied the equilibrium concentration of ATP and of $K^+$ in frog muscles after exposure to 11 poisons of widely diverse toxicological effects. Their data show that the predicted linear relationship between ATP concentration and $K^+$ concentration in the cell does indeed hold. In addition, according to theory, ATP is responsible for water polarization in the cell. It has been shown that as ATP concentration fell in a specific frog muscle experiment where 0.2 mM icdoacetate was used, the changes of $K^+$ and $Na^+$ concentration are like mirror images as expected ($K^+$ down, $Na^+$ up). In this experiment a parallel

course of change of sucrose concentration and $Na^+$ concentration has also been recorded indicating that the $Na^+$ taken up is dissolved in the depolarizing cell water (q-value change) and not stoichiometrically replacing adsorbed $K^+$. Were it otherwise, and the cell water remains unchanging, then sucrose which cannot replace cationic $K^+$ would have remained unchanged in concentration (for discussion of this experiment see Ling, 1992a, text reference, pp 190-192). Note the different action of ouabain on the cooperatively linked $\beta$- and $\gamma$-carboxyl groups. Ouabain transforms the ion binding sites from a $K^+$-preferring state into a $Na^+$-preferring state without changing the water structure. $Na^+$ is stoichiometrically replacing adsorbed $K^+$ (see e.g., Edelmann 1986a, text reference) and the q-values of large solutes in cell water are not changing (for experimental evidence see Ling, 1992a, text reference, pp 172-178).

G.M. Roomans: You propose the study of water structure in sections of frozen-hydrated muscle by e.g., electron diffraction. Do you have an idea of the size of the different water domains involved (structured vs. free) and is the resolving power of electron diffraction in frozen-hydrated sections sufficient to discriminate between these two domains?
Author: Under the assumption that water molecules are less and less ordered with increasing distance from the polarizing protein surface it is very unlikely that the different states of order can be detected within a subcellular compartment of a frozen hydrated section. One might however expect differences in the electron diffraction patterns of differently hydrated perfectly vitrified preparations which contain different fractions of well ordered water dipoles and possibly differences when comparing healthy with deliberately killed muscles.

Reviewer IV: There are numerous pharmaceutical agents used in clinical medicine whose mechanism of action is known to be on the $Na^+$,$K^+$-ATPase pump. These agents are therapeutically very effective and have a specific known mechanism. How can the AIH explain the therapeutic effects of such drugs?
Author: The explanation of the mechanism of therapeutically effective drugs given by the MPT is by no means a precise description of the molecular mechanisms which are e.g., responsible for the observable change in ATPase activity and for the observable redistribution and permeability changes of ions like $Na^+$, $K^+$, $Ca^{++}$, and water. For instance, it has been shown that the perfused squid axon contains functioning $Na^+$,$K^+$ ATPase and is able to increase $Na^+$ efflux by addition of ATP (!) and the efflux is sensitive to ouabain (!); however, a net $Na^+$ efflux against an electrochemical gradient could not be observed (for review see Ling, 1984, p 127, text reference). This experiment shows that it is not justified to call the $Na^+$,$K^+$-ATPase a pump and it shows that the descriptive explanation of the MPT of drug action (e.g., ouabain, strophanthin) in terms of MPT pictures is not reflecting basic understanding. According to the AIH, ion permeability changes and ion redistribution phenomena are caused by conformational changes of the ATP-ATPase system and connected proteins with the result of selectivity changes of ion adsorbing sites and changes of water polarization.

Reviewer IV: You have cited EPXMA studies on $K^+$ distribution in skeletal muscle as demonstrating that the location of $K^+$ is consistent with the AIH. Since quantitatively EPXMA measures mass fraction (mass per mass) and not concentration (mass per volume) the quantitative relationship to a subcellular component may be a theoretical construct and not a real functional relationship. What direct evidence is there that $K^+$ is actually bound to proteins located in the A band of skeletal muscle?
Author: EPXMA studies allow the determination of local water and dry mass fractions. Therefore the concentration of an element in a subcellular area can be calculated (see e.g., von Zglinicki, 1988, text reference). The experimental results reviewed in this paper show that $K^+$ and other alkali-metal ions and $Tl^+$ are preferentially localized in regions where most $\beta$- and $\gamma$-carboxyl groups are located (A band, Z line). One may argue that the localization may be due merely to the presence of fixed anionic charges and that the cations are confined not as adsorbed ions but merely as free counter ions. This possibility was ruled out, when the critical difference between the attribute of ions within the ion cloud which balances fixed opposite charges and the attributes of ions adsorbed on discriminating sites were examined and tested experimentally (Ling and Ochsenfeld, 1966, text reference; see discussion by Ling, 1977, text reference). Direct evidence for selective ion adsorption in the A band and Z line was provided by the "staining experiments" with freeze-dried preparations (Edelmann, 1980b, 1981, 1984a, 1986a, text references).

Reviewer IV: There are many other cell types - including plant cells - that are thought to depend on membrane pumps to maintain their distribution of cations necessary to develop a membrane potential according to the MPT. These cells do not have

structures like skeletal muscle A bands and do not exhibit observable subcellular cation distributions associated with protein structures. How does the AIH explain these sort of EPXMA findings?

**Author:** Such findings are neither suited to establish the nature of the cellular electrical potential (MPT: membrane potential, AIH: phase boundary potential) nor do they prove or disprove cellular ion binding. If it is not possible to observe subcellular cation distributions associated with protein structures with EPXMA it is of course impossible to test AIH predictions as has been done with muscle. However, one may try to detect ion adsorbing sites by using the reported technique of staining sections of freeze-dried and embedded preparations with alkali-metal ions. It should be emphasized that of course "what is true of *E. coli*, must be true of elephants" (Monod and Jacob, 1961). But EPXMA is not the only tool to study the physical state of ions and water. Indeed with a variety of other methods and tools the various predictions of the AIH have been tested and affirmed in cells ranging from animal cells to plant cells and microbial cells (Ling, 1962, 1984, 1992a, text references).

T. von Zglinicki: The reader of a review pro and con MPT must expect that recent important developments in both fields are fairly covered. This minimum requirement has been missed by nearly one decade. The author states that the molecular working mechanism of the $Na^+,K^+$-pump is unknown by citing a reference which is more than 20 years old (Glynn and Karlish, 1975, text reference). Within the last five years, pumps have been cloned to a large extent, and the expression of mutant forms carrying single point mutations in well-defined codons has been shown to abolish the ouabain- or amiloride-sensitivity of the respective cells. So, a contemporary experiment would be to express those mutations in muscle and see whether ion changes can be fully explained by the mutation or whether additional mechanisms might play a role. On the other hand, all pro-AIH experiments done by the author were published until 1991 and no new aspects have been added.

**Author:** With the information reviewed in this paper it is not justified to state that pumps have been cloned because not pumps but $Na^+,K^+$-ATPases have been cloned and because the proof is missing that the $Na^+,K^+$-ATPase is pumping $Na^+$ or $K^+$ (Ling and Negendank, 1980, text reference). Even today the molecular mechanism of the working of the postulated pump is unknown and it is justified to cite an old reference pointing to this problem. I have cited this reference for pump-believers but having in mind the profound experimental evidence for the view that the pump idea is not tenable (it violates the Law of Conservation of Energy - a fact first pointed out in detail by Ling, 1962, text reference, for most up-to-date details see Ling, 1997, linked page: <http://www.gilbertling.org/lp6b.htm>) I conclude that the working mechanism of the pump will never be discovered. (It has been mentioned that active transport across bifacial cells like frog skin or intestinal epithelium is an established fact; it is also accepted by followers of the AIH that the $Na^+,K^+$-ATPase is playing an important role in this true active transport process but even here the ATPase *per se* is not a pump). The successful cloning of the $Na^+,K^+$-ATPase has not yet changed this picture but I appreciate the suggestion to think about new acceptable test experiments. The statement that no new aspects have been added in this review is not true. I have discussed at some length the properties of *extroverts* and *introverts* and a new quantitative theory of water polarized in multilayers and its experimental verification (Ling, 1992a, 1993, text references), and I have derived new proposals for future investigations of the ion and water problem by using cryotechniques and electron microscopy.

T. von Zglinicki: The data of the pro-AIH experiments done by the author rest on the assumption that ion distributions measured in suboptimally frozen and most often freeze-dried or freeze-substituted tissue samples faithfully resemble the *in-vivo* distribution of ions on a 1 $\mu$m-scale. However, as the author discusses himself, ion precipitation onto macromolecular charges is likely to occur during preparation. Because this issue has, to my knowledge, never been settled, the argument of the measured ion distribution following the distribution of proteins in muscle is too weak to support the far-reaching conclusions of the author.

**Author:** I have discussed in an earlier paper the problem of ion precipitation (a strong binding) during cryo-preparation (Edelmann, 1994, text reference). It was shown that precipitation only occurred after "incorrect" freeze-drying (too short at low temperature) and that the ion distribution is similar in properly freeze-dried and in frozen-hydrated preparations which both show a well preserved ultrastructure without visible ice crystal damage. Since it is accepted that "displacement of ions over distances larger than the ice crystal damage as seen in sections can most likely be safely neglected" (von Zglinicki, 1991, p. S87) it must be concluded that the observed ion distribution is close to the *in-vivo* distribution. Whereas the molecular mechanism of ion association to muscle proteins cannot be deduced by

General conclusions: Teleonomic mechanisms in cellular metabolism, growth, and differentiation.

microanalytical detection of ion distributions alone (see discussion with reviewer IV) the conclusion concerning the state of water in muscle (see text) is inevitable. It must be noted that further far-reaching conclusions are by no means based solely on detected ion distributions in muscle but rather on many different findings of specially devised test experiments which confirm the AIH in a consistent manner.

### Additional References


Braben D (1996) The repressive regime of peer-review bureaucracy? Physics World, November 1996, 13-14.

Cameron IL, Fullerton GD, Smith NKR (1988a) Influence of cytomatrix proteins on water and on ions in cells. Scanning Microsc 2: 275-288.

Cameron IL, Hunter KE, Fullerton GD (1988b) Quench cooled ice crystal imprint size: A micro-method for study of macromolecular hydration. Scanning Microsc 2: 885-898.

Clegg JS (1984a) Intracellular water and the cytomatrix: Some methods of study and current views. J Cell Biol 99: 167s-171s.

Clegg JS (1984b) Properties and metabolism of the aqueous cytoplasm and its boundaries. Am J Physiol 246: R133-R151.

Clegg JS, Drost-Hansen W (1991) On the biochemistry and cell physiology of water. In: The Biochemistry & Molecular Biology of Fishes. Hochachka PW, Mommsen TP (eds). Elsevier Science Publishers, Amsterdam. Vol. 1, pp 1-23.

Edelmann L (1982) Comments on Kernan's misrepresentation of the association-induction hypothesis in his book "Cell Potassium". Physiol Chem Phys 14: 215-218.

Gulati J, Ochsenfeld MM, Ling GN (1971) Metabolic cooperative control of electrolyte levels by adenosine triphosphate in the frog muscle. Biophys J 11: 973-980.

Hazlewood CF (1979) A view of the significance and current understanding of the physical properties of cell-associated water. In: Cell-Associated Water. Clegg J, Drost-Hansen W (eds). Academic Press, New York. pp 165-259.

Hazlewood CF (1995) Water movement in diffusion in tissues. In: Diffusion and Perfusion: Magnetic Resonance Imaging. Le Bihan D (ed). Raven Press, New York. pp 123-126.

Kasturi SR, Hazlewood CF, Yamanashi WS, Dennis LW (1987) The nature and origin of chemical shift for intracellular water nuclei in Artemia cysts. Biophys J 52: 249-256.

Ling GN (1997) Why science cannot conquer cancer and AIDS without your help? WWW address: http://www.gilbertling.org/

Monod J, Jacob F (1961) Cold Spring Harbor Symp Quant Biol. 26: 389, p.393

Von Zglinicki T (1991) Reliability of intracellular water and ion distributions as measured by x-ray microanalysis - a review. Scanning Microsc Suppl 5: S85-S93.


389-401