THE STATE OF SODIUM AND WATER

IN

SINGLE STRIATED MUSCLE FIBERS

by

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ABSTRACT

Cation sensitive glass microelectrodes were inserted into single striated muscle fibers of the giant barnacle, Balanus nubilus, to measure directly the activities of sodium and potassium in the myoplasm. The total sodium and potassium content of the individual experimental fibers was determined by flame photometry. From these measurements, the percentage of sodium in the fiber which did not affect the microelectrodes and the percentage of water in the fiber which was not available to act as solvent for the potassium ions were calculated. The minimal percentages of "bound" sodium and water were 84% and 42% respectively. It was hypothesized that a significant fraction of this "bound" sodium was involved in ion pair formation with carboxyl moieties on the myosin molecules which comprise the thick filaments, and experiments were designed to test this hypothesis.

In the second series of experiments, the activities of sodium, potassium and hydrogen in the myoplasm were measured as the temperature of the solution bathing the fibers was increased from 7 to 40°C. An irreversible shortening occurred in all fibers between 37 and 40°C. When the fibers shortened in a sodium free Ringer solution, the mean activity of sodium increased by 130%, the mean activity of potassium remained relatively constant, and the pH decreased from 7.17 to 6.77. These experiments provided strong evidence that sodium is bound to myosin in the living fiber, for extracted myosin is known to denature at 37°C and release its associated alkali metal cations.

In the third series of experiments, the optical density, O.D., of the single striated muscle fibers was measured at 50 mμ intervals between

450 and 850 mμ. At all wavelengths, the O.D. decreased markedly when the normal Ringer bathing solution was replaced by sodium free sucrose Ringer. For example, at 850 mμ the O.D. of the fibers, relative to the initial value in normal Ringer, decreased from 1 to 0.21 ± 0.06 in 25 minutes. The corresponding increase in the transmittance, T, (O.D. = -log T) was from 5% to 55%. This change in O.D. could be reversed by returning the normal Ringer bathing solution to the bath. Large, reversible decreases in O.D. were also observed when potassium and tris were used as substitutes for sodium. These changes in O.D. are explained by the theory of light scattering if it is assumed that sodium is bound to the main scattering centers in the myoplasm, the thick filaments. When the fibers were bathed in sodium free, lithium substituted Ringer, a small reversible increase in the O.D. was observed, which may indicate that lithium is complexed more strongly than sodium to the binding sites on the thick filaments.

In the final series of experiments, the number of sodium and potassium ions "bound" to the contractile proteins in a glycerinated fiber was measured. The free concentrations of hydrogen, sodium and potassium were maintained at values similar to those found in an intact fiber. The results indicated that substantial binding of both sodium and potassium occurred, and that proportionally more sodium than potassium ions were "bound". If the results are extrapolated to the intact fiber, they imply that about as much sodium is "bound" to the contractile proteins as is free in the myoplasm. This amount of "bound" sodium is sufficient to explain the results of the denaturation and light scattering experiments, but insufficient to account for the anomalously low activity of sodium in the myoplasm, as measured by a sodium sensitive microelectrode. Thus, it was

concluded that either some factor must enhance the binding of sodium to the contractile proteins in a living cell, or that sodium must be sequestered in organelles which are destroyed by the glycerination process.

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CHAPTER I

HISTORICAL INTRODUCTION

Shortly before his death, Sir Isaac Newton remarked: "I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary whilst the great ocean of truth lay all undiscovered before me." What was true of 17th Century mathematics and physics (and Newton was 17th Century mathematics and physics) is also true of 20th Century biology. Much time has been spent playing with cells and organelles, but for the first half of this century, the sea-like environment of these cells and organelles was almost completely ignored. Water was considered merely as the inert, intracellular medium in which the biochemical reactions of the cell occured. In the last decade, however, many investigators recognized the importance of understanding the role of water in cell physiology and biochemistry. As Szent-Györgyi has stated: "water is not only the <u>mater</u>, mother, it is also the matrix of life, and biology may have been unsuccessful in understanding the most basic functions because it focussed its attention only on the particulate matter..." (1).

It is not fair to say that water was completely ignored by the earlier biologists. There are sporadic references in the literature to the possibility that water in the cytoplasm is not in the same physical state as normal liquid water. Over 60 years ago, Overton (2) observed that a muscle swelled to much less than twice its initial weight when immersed in a solution of half the initial osmotic pressure, and concluded that a sub-

stantial fraction of cellular water was osmotically inactive. Twenty years later, Rubner (3) estimated the fraction of water which could not be frozen in a muscle at -20° C, and deduced that 23% of the water in frog muscle was "bound". In 1930, however, Hill (4) concluded from vapor pressure measurements that less than 4% of the water in frog muscle was "bound". Interest in the state of water in cells remained dormant for another 20 years. It is interesting to note that if an error in Hill's calculations is corrected, his data predict that about 30% of the water in frog muscle is "bound" (5).

In recent years, the interest of chemists in the structure of water has stimulated the interest of physiologists and biochemists in this biologically ubiquitous molecule. In the last two years alone, several conferences have been held (6, 7, 8) and books written (9, 10, 11) on the importance of the state of water in the living cell.

Most physiologists have assumed that all the alkali metal cations, as well as the water, in a cell exist in a free state. Many years ago, however, several investigators suggested that the selective potassium ion accumulation of cells may be due to binding rather than a membrane phenomenon (12, 13, 14). In 1929 Höber (15) critically analyzed this possibility. He concluded that only the proteins in a cell exist in large enough quantities to complex sufficient ions to explain the selective accumulation of potassium by an absorption mechanism. For this reason, he examined the available data on complexing of ions by extracted proteins. He observed that the amount of ion binding by proteins in solution was small. This observation is still valid, with the exception of a few proteins like myosin (16). He also observed that there was no evidence of a marked preference of proteins for potassium over sodium ions. Thus, he concluded that

the binding of ions to proteins could not explain the selective accumulation of potassium in living cells.

In spite of Höber's analysis, there exist today groups of investigators in Australia (17-20), Russia (21-23) and the USA (24-31) which do not accept the assumption that the intracellular ions in general, and the potassium ion in particular, are free in the cytoplasm. Ernst (32, page 311) has listed several other investigators who prefer, in one form or another, a sorption theory of ion accumulation to the more generally accepted membrane theory. The proponents of the sorption theory base their hypotheses not so much on experimental evidence for ion binding as on criticisms of the membrane theory and the auxilliary postulates required by this theory. The necessity of postulating a wide variety of "ion pumps" located in the membrane, for example, leads to serious contradictions, as Troshin (21) and Ling (29, 30) have pointed out. These groups have speculated that most of the potassium, but not the sodium in the cell exists in a complexed or associated state. This speculation has never been widely accepted. Conway (33), for example, has criticized the theory, directing his criticisms mainly at Ling. The microelectrode experiments reported in this thesis also contradict the sorption theory, for they demonstrate that the activity of potassium in the myoplasm of striated muscle fibers is actually higher than would be calculated by assuming that all the water and potassium ions are free in the myoplasm. The mere rejection of the sorption theory does not, of course, remove the criticisms of the membrane theory. These criticisms will be discussed further in Chapter IX.

This investigator certainly does not accept the sorption theory, but wishes to stress that the advocates of this theory have performed an

important function. They have forced biologists to examine more thoroughly the question of ion association in living cells. The theoretical aspects of the association of the alkali metal cations with various anions have been studied by Eisenman (34, 35) and Ling (25). The development of cation sensitive microelectrodes by Hinke (36) and the application of a nuclear magnetic resonance technique by Cope (37) has allowed biologists to study directly for the first time the binding of sodium and potassium in living cells. The results of these studies (5, 36-40) indicate that a substantial amount of ion pair formation does occur in the myoplasm of striated muscle fibers, but that it is sodium and not potassium which is preferred by the biological fixed charge system.

CHAPTER II

PHYSICAL CHEMISTRY OF THE BINDING OF WATER AND THE ALKALI METAL CATIONS

A. Water

Introduction. This section consists of a brief review of the structure of water and the effects of various solutes on this structure. The possible effects of proteins and membranes on the structure of water in the cytoplasm of a living cell will be considered, as will the methods of examining these structural changes.

The Structure of Water. The structure of water was first discussed in the modern crystallographic sense by Bernal and Fowler (1) in 1933. They postulated that extensive hydrogen bonding occurs between water molecules, and considered water as a disordered solid having an irregular four co-ordinated structure. The view that water is merely a broken-down form of the ice lattice, with the length of the hydrogen bonds increased, is supported by a great deal of experimental evidence. The most direct evidence comes from X-ray scattering measurements (2-8). Morgan and Warren (2) were the first to show that for short periods of time each water molecule has four nearest neighbours, and, at temperatures below 30° C, a second set of twelve nearest neighbours. The distance at which these nearest neighbours are found is compatible with the view that water has a tetrahedral structure similar to ice.

In 1957, Frank and Wen (9) presented what is now the most generally accepted model of water. They based their model on the hypothesis that the formation of hydrogen bonds in water is a co-operative phenomenon. This is a reasonable hypothesis because the hydrogen bond has a partial

covalent nature (10). They argued that when one hydrogen bond forms in water, the formation of neighbouring bonds is encouraged and stabilized. Similarly, they argued that when one bond is broken by thermal agitation, the entire group tends to break up. Thus, they picture water as consisting of minute "flickering clusters" of ice-like groups surrounded by non-hydrogen-bonded molecules (11, Fig. 1). They did not specify the exact molecular arrangement within these groups, but Nemethy and Scheraga (12, 13) made the reasonable assumption that the tridymite-like structure of normal ice I occurs frequently. On the basis of this assumption Nemethy and Scheraga (12, 13) made a detailed statistical mechanical analysis of the structure of water. They concluded that at 20° C the average cluster contains about 60 molecules and that at this temperature about 70% of the water molecules are in the clusters. These numbers increase as the temperature is lowered, and vice versa. A minimal estimate of the life time of the clusters can be made from experimental data. The clusters must exist long enough to be detected by X-ray (2) or Raman (14, 15) techniques; that is, about 10^{-11} - 10^{-12} seconds. It is more difficult to make a maximal estimate of the life time of the clusters. If, as Frank (16, 17) believes, the dielectric relaxation time of water is equal to the half life of the clusters, the life time of the clusters is 10^{-10} - 10^{-11} seconds (18). This is 100 to 1000 times the period of a molecular vibration, hence the clusters have a meaningful existence.

It should be stressed that many other models for the structure of water exist. Kavanau (19, pages 178-190), in a terse and lucid manner discusses the "vacant lattice point" model of Forslind (20, 21), the "water-hydrate" model of Pauling (22, 23) and the "distorted bond" model of Pople (24). He also lists over a dozen reviews of still other models for the

structure of water. None of these models will be discussed here. For this thesis, it is sufficient to note that pure water does have a structure. Also, the flickering culster model is the most highly developed, and appears to explain all the experimental data available. The values for the free energy, enthalpy and entropy calculated by Nemathy and Scheraga (12, 13) agree very well with the experimental data. The values they calculated for the heat capacity agree reasonably well with the experimental data, as do the calculated curves for the radial distribution function. The maximum in the density of water at 4^{o} C may also be predicted qualitatively from the theory. The infra red studies of Buijs and Choppin (25) support the model, and it is significant that these measurements were made after the formulation of the model. The temperature dependence of viscosity (26) agrees extremely well with Nemethy and Scheraga's treatment of the model. An explanation exists for the fact the energy of activation for self diffusion, viscous flow, dielectric relaxation or structural relaxation for excess ultrasonic absorption has approximately the same value (16, 17, 27). In terms of the model, most of the energy of activation for these processes is required to break down the initial structure; little energy is required to reorient the molecules. Finally, one can logically explain why non-polar solutes enhance the ice-like nature of water in terms of this model. This last fact leads to a discussion of the effect of solutes on the structure of water.

The Effect of Non-polar Solutes on the Structure of Water. Consider first the evidence that the structure of water is enhanced when non-polar solutes are added. If the solution resulting from the addition of a non-polar solute to water was ideal, one would expect the changes in volume and enthalpy to be zero, and the changes in entropy and free energy to

correspond to those for ideal mixing. For non-polar solutes, however, the changes in volume and enthalpy are negative and there is a very large negative excess entropy change over the entropy of ideal mixing. This leads to a low solubility. Frank and Evans (28) explained the negative enthalpy and excess negative entropy terms by postulating that the non-polar molecules enhance the structure of water. In other words, the flickering clusters are stabilized. This tends to be confirmed by the fact the dielectric relaxation time is lengthened in aqueous solutions of non-polar molecules (16).

Why should non-polar solutes stabilize the "flickering clusters"? Frank and Evans (28) offered the rather heuristic explanation that non-polar solutes do not transmit disruptive electrical influences well because of their low polarizability, hency stabilize the structure. Nemethy and Scheraga (12, 13) and Scheraga (11) offered a more precise explanation. They argued that a water molecule in pure water has five energy levels available to it. These energy levels correspond to molecules with zero, one, two, three or four hydrogen bonds. A molecule with four hydrogen bonds can accept a non-polar molecule as a fifth neighbour. Thus, this energy level is lowered because of the van der Waals energy of interaction. An unbonded water molecule, on the other hand, already has a high co-ordination number and can acquire a non-polar neighbour only at the expense of a water molecule. This implies that a dipole-dipole interaction will be replaced with a much weaker van der Waals interaction. Hence, the energy level for the unbonded water molecules in contact with non-polar solutes is increased. The energy levels for molecules involved in one, two or three hydrogen bonds are raised for the same reason (11, Fig. 8). If a Boltzmann distribution of the water molecules between the 5 energy levels is assumed, it is apparent that the addition of non-polar solutes to water shifts more

molecules into the lower (four hydrogen bonds) energy state. Since the non-polar solute fills a space which would be empty in an ordinary ice-like cluster, there is a decrease in volume. The hydrogen bonded water molecules in the first layers about a non-polar solute are generally known as "icebergs" (28).

The Effect of Ions on the Structure of Water. Ions have a dual effect on the structure of water. The first effect is on the water molecules close to the ion. These are generally regarded as being immobilized, polarized and compressed by the interactions of the dipole moment with the strong electric field of the ion (1, 3, 9, 28, 29, 30, 31). The term "immobilized" implies that the water molecules bound to cations like cesium or lithium spend about 10^{-8} and 10^{-9} seconds respectively attached to the ions (32). It is apparent that the ion imposes a different type of order on the closest water molecules than the order inherent in the normal water. Nevertheless, the ion does increase the order of these nearest neighbour water molecules. One would expect the ordering effect to be greatest for small and multivalent ions. Much experimental evidence supports the idea that small ions increase the net order of water. Perhaps the most direct evidence is the fact that salts like LiF increase the viscosity of water (33). Other evidence comes from measurements of the entropies of hydration (28), apparent molal heat capacities (34) and the temperature dependence of the limiting diffusion constant (35).

A manifestation of the dual effect of ions on water is the fact that monovalent cations larger than potassium (and most anions) disrupt the structure of water. They decrease the viscosity of water (33). The entropy loss which occurs when a KCl molecule is dissolved in water is less than

when two argon atoms are dissolved, even though they both have the same electronic structure (28, 36). Other evidence for the net structure breaking activity of large ions comes from measurements of the ion mobilities (3), measurements of the self-diffusion coefficient of water in electrolyte solutions (35) and the fact large ions reduce the relaxation time for the dielectric constant (37).

The structure breaking effect of larger ions is readily explained in terms of the "flickering cluster" model. There exists two regions of ordered water molecules about an ion. Far from the ion there exists a region with the structure of normal water. In the immediate vicinity of the ion the water molecules are oriented by the strong, spherically symmetric electric field of the ion. The buffer zone, or intermediate region between these two states of competing and incompatible order is itself in a state of chaos. Thus, what is often true in international politics is true in chemistry. The lack of order of the water molecules in this intermediate region explains the structure breaking effect of the larger ions. The smaller ions organize water molecules beyond the first layer of water, hence their net effect is to enhance the structure of water slightly. Further discussion of the structure breaking action of ions can be found in the papers of Frank and Evans (28), Gurney (3), Frank and Wen (9) and Harris and O'Konski (38). The water which is oriented and bound by the electric field of ions is often designated as "soft ice", in contrast to the term "icebergs", which is used to describe the water organized by non-polar solutes.

The Effect of Proteins and Membranes on the Structure of Water. The structure of water will also be influenced if a solute contains sites which can interact with water molecules through the formation of hydrogen

bonds. Hydrogen bonding will occur at the peptide linkages of proteins. At most, four water molecules can be bound by each peptide group (39), but this amount of interaction could only occur in unfolded proteins, where the peptide linkages are free to interact. In an α-helix, all of the peptide linkages are involved in the maintenance of the molecular structure. In muscle the α-helix content of the proteins is high (40), hence not much direct water binding to the peptide linkages would be expected. The amount of water bound directly to macromolecules via ion-dipole, hydrogen bonds and the weaker dipole-dipole interactions may thus be expected to be small. This does not mean, however, that these macromolecules do not organize relatively large amounts of water.

The consideration of the organization of water about a protein entails the consideration of the geometry of the protein, and how it will fit into the water lattice. For example, Berendsen and Migchelsen note that "Backbone structures able to form H-bonds to water will have structure-breaking or structure-promoting effects, depending on the geometry of the hydrogen-bonding sites. If the geometry is such that the sites, to which water may be bound, form an array fitting to an ice-I structure, a structure-promoting influence is to be expected. The same may be true if other regular water structures could be fitted to the hydrogen bonding sites. With hydrophobic backbones similar effects might occur if short polar side-chains repeat in a pattern fitting to a regular water lattice. The effects will be stronger for rigid backbones or side chains." (41). Collagen is an example of a molecule with such a structure. The axially repeating distance of the three fold helix is exactly six times the expected repeating distance of molecules in chains of water (42). Thus, one would expect collagen to organize water, and NMR studies indicate that chains of water molecules are

formed in the fiber direction (41, 43).

Water may also be oriented at the interfaces of extended surfaces, and there is some evidence that these surface zones are tens and hundreds of molecules deep rather than monomolecular as commonly assumed. The water lattice in a clay crystal, for example, appears to acquire an increased order and rigidity at distances of up to 300 Å away from the surface of the clay (44, 21).

The extent to which water is organized about most proteins and biological membranes is still unknown. Initial dielectric and NMR measurements on macromolecular solutions supported the concept that water is immobilized into thick, ice-like hydration crusts for large distances away from macromolecules (45, 46). Improvements in the NMR technique and a recognition that the dielectric properties of macromolecules could largely be explained in terms of the polarization of the diffuse double layer, however, have largely invalidated the concept of long range immobilization; on the other hand, most recent NMR experiments do indicate that the net time which solvent water molecules spend in a given orientation is lengthened to varying degrees in solutions of macromolecules (19, pages 207 - 217). After a thorough literature search, Kavanau concluded that biological membranes and macromolecules "are encased in a thin crust of bound water molecules at least one molecule thick" (19, page 217).

The State of Water in the Living Cell. The term "bound water", as used above, is rather ambiguous. As noted in Chapter I, many investigators are now interested in the fraction of "bound water" in the living cell, but each seems to have his own definition of the term. In practice, the term is defined by the technique utilized to measure the fraction of "bound water".

This is apparent if one considers that the values obtained for the water "bound" to a given protein by different techniques are themselves quite different (47).

In this thesis, the term "bound water" will refer to the fraction of water in the cell unavailable to act as solvent for the intracellular solutes. It should be stressed that this definition is as arbitrary as the other definitions available at present and that it is not meant to imply that water in a living cell is physically partitioned between two and only two states. In spite of the arbitrary nature of the definition, an accurate measurement of the fraction of "bound water" is important because of its relevance to the osmotic, permeability and transport measurements typically made by physiologists.

It seems unlikely that NMR (48) or desorption (49) measurements will yield much information about this fraction of "bound water", for organization and solute exclusion are not synonymous. In terms of the definition, however, this quantity can be measured directly. All that is required is a knowledge of the free and total concentrations of the solute in the cell. If the free concentration is greater than the total concentration, the difference may be attributed to the "binding" of water, presumably to macromolecules and membranes. It must of course be assumed that the solute itself is neither bound nor compartmentalized within the cell.

Defined in this manner, the fraction of "bound water" depends on the nature of the solute as well as on the state of water in the cell. A small, polar solute like urea, which is capable of forming strong hydrogen bonds, would be expected to be soluble in almost all of the intracellular water. Conversely, a large, non-polar molecule would be expected to be ex-

cluded from most of the organized water in the cell. It would even be dangerous to assume that all the ionic species make use of the same fraction of water. Ice I, for example, is known to exclude sodium more than potassium (50).

As potassium is the major cation in muscle fibers, a knowledge of the fraction of water unavailable to act as solvent for this ion is of special importance. The development of glass microelectrodes sensitive to potassium (51) made possible a direct measurement of the activity of potassium in the myoplasm of a large single muscle fiber (52, 53). When these measurements were coupled with accurate measurements of the total concentration of potassium in the same single muscle fiber, an estimate of the fraction of "bound water" in the cell could be made. To foreshadow the results, which are discussed in the following chapters, it may be noted that Hearst and Vinograd (54, 55) measured the fraction of water in a DNA solution which excluded an alkali metal salt. From their density gradient studies, they concluded that a region consisting of four layers of water molecules about the T-4 bacteriophage DNA molecule completely excluded a lithium silicotungstate salt.

B. The Alkali Metal Cations

Introduction. This section is concerned with evidence that the alkali metal cations can form ion pairs with charged groups on macromolecules. First, the binding of these ions to polyelectrolytes and ion exchange resins is considered. Next two theories of cation selectivity are briefly discussed. Finally, the evidence that the alkali metal cations form complexes with proteins is appraised.

Before any discussion can commence, the definition of an ion pair must be considered. The term is not as ambiguous as the term "bound water", but is sufficiently abstruse to warrant comment. If two oppositely charged ions are small or highly charged and also close together, the energy of the electrical attraction will be greater than the thermal energy, and the ion pair will survive a number of collisions with solvent molecules. Bjerrum (56) investigated the problem of exactly how close a given pair of ions must be before they may be considered an ion pair. He concluded that the average effect of ion pair formation is best represented if two ions are considered to form an ion pair when they come closer together than a distance $r_{min} = (z_+ z_- q^2)/(2DkT)$. In the definition, z_+ and z_- represent the charges on the ions, q the electronic charge, D the dielectric constant, k Boltzmann's constant and T the absolute temperature. At this distance the mutual electric potential energy is equal to 2kT. The distance was chosen because the probability of finding an oppositely charged ion about any given ion has a minimum at r_{min}. Robinson and Stokes (57, Chapter 14) discuss briefly the mathematical basis of this definition. Monovalent electrolytes in an aqueous solution at 25° C have a value of $r_{min} = 3.57$ Å. Thus, if the sum of the radii of the ions of a monovalent electrolyte is less than this value, ion pair formation will occur to a certain extent. If the ions of a monovalent electrolyte have diameters greater than this value, some form of the Debye-Hückel theory should be valid.

Several criticisms have been made of Bjerrum's theory, but only two need be noted here (58). The first criticism is that the theory counts as ion pairs some ions which are not in physical contact, and as Bjerrum himself notes, "this definition is rather arbitrary" (56). The second criticism is that the distance of closest approach of the ions predicted from the

Bjerrum theory varies from solvent to solvent. This implies that an ion pair can contain solvent molecules between the two ions. A brief discussion of improvements to Bjerrum's definition and of other definitions of ion pairs may be found in a book by Rice and Nagasawa (59, pages 441-446).

Polyelectrolytes and the Alkali Metal Cations. The alkali metal cations form no strong or even moderately strong complexes with most common small molecules and ions. Exceptions are the complexes formed with strong chelating agents like EDTA (60) and uranyl diacetic acid (61). The extent of ion pair formation with carboxyl or phosphate moieties on other small molecules is certainly very slight. It might therefore be suspected that the alkali metal cations are essentially free in a solution of polyelectrolytes containing carboxyl or phosphate groups. Under certain conditions, however, exactly the converse is true.

The best known method of determining the extent of ion binding is that of measuring the transference numbers of the polyions and the counterions using radioactive tracers (62). Measurements on polyacrylic acid (a polycarboxylic acid) demonstrated that in a 0.0151 N solution at 50% neutralization about 50% of the sodium in the system was bound (62). The percentage of sodium ions bound was a function of the degree of neutralization of the polyelectrolyte.

A diffusion method for determining the amount of counterion binding by polyelectrolytes was also developed by Wall and his coworkers (62). The theoretical basis for this technique is given in detail by Crank (63, pages 121-122). It must be assumed that the process of ion pair formation proceeds quickly compared to diffusion. If this is so, local equilibrium may be assumed to exist between the free and the bound components. For

simplicity, a linear adsorption isotherm may be assumed. That is, the concentration of the immobilized substance, S, is assumed to be directly proportional to the concentration of the substance free to diffuse, C.

$$S = RC \quad \text{.....[1]}$$

where R is a constant. Other cases are discussed by Crank (63). The usual equation for diffusion in one dimension (Fick's Law) is then modified to allow for adsorption, and becomes

$$dC/dt = D\, d^2C/dx^2 - dS/dt \quad \text{.....[2]}$$

if the diffusion coefficient, D, is assumed to be constant. Eqn. [1] may be substituted into Eqn. [2] to yield

$$dC/dt = D/(R + 1) \cdot d^2C/dx^2 \quad \text{.....[3]}$$

which is the normal form of the diffusion equation with D replaced by $D/(R + 1)$. Thus, the extent of binding can be calculated from a measurement of the self diffusion coefficient. The amount of sodium bound to polyacrylic acid calculated by this technique was found to be almost identical to the amount calculated by the transference number technique (62).

The viscosity, osmotic pressure, turbidity and electrophoresis characteristics of a polyelectrolyte solution depend on the effective charge on the macroion. Thus, measurements of these parameters may yield valuable information about the extent of ion binding in the solution. Strauss and his coworkers (64-70, see especially 65, 68-70), for example, used electrophoresis and membrane equilibrium techniques to determine that the binding of the alkali metal cations to polyphosphates increases in the order Li>Na>K.

The selectivity with which the alkali metal cations are bound is

interesting, but it is the magnitude of the binding that is really surprising. The extensive binding of the alkali metal cations is of course partially due to the high negative charge on the polyion, which enhances the concentration of cations in the double layer surrounding it. A correction for this factor may be applied by considering the corrected association constant

$$K = [MP]/[P][M]_{eff} \qquad \text{.................................[4]}$$

where [MP] represents the concentration of the ion pairs, [P] the concentration of the free sites on the polyion and $[M]_{eff}$ the effective concentration of the cation M near the polymer chain. This concentration is given by the Boltzmann relation

$$[M]_{eff} = [M] \exp(-q\Psi/kT) \qquad \text{.............................[5]}$$

where [M] represents the cation concentration far from the polymer chain, q the charge on the cation, k the Boltzmann constant, T the absolute temperature and Ψ the electrostatic potential at the surface of the polyelectrolyte. (To apply this correction, Ψ must be assumed to equal the zeta potential, which may be calculated from the electrophoretic mobility.) Even with a correction for the Boltzmann factor, the extent of binding is extremely high. The association constants for the phosphate polyions and the alkali metal cations range from about 1 to 5 moles^{-1}.

The experiments of Strauss argue against the earlier concept that ions are attracted to a polyion merely because of its high net charge (71) and are trapped in a region where $|q\Psi/kT|>1$. Additional evidence that true ion pair formation occurs in polyelectrolyte solutions is discussed by Rice and Nagasawa (59, pages 450-455).

It was noted above that the polymerization of phosphate or carboxylate monomers into a polyelectrolyte enhances the binding of the alkali metal cations to these moieties. It is interesting that an analagous phenomenon has been known to colloid chemists for over 30 years. When certain paraffin chain cations form micelles at a critical concentration (72), the number of anions bound to these cations is markedly enhanced (73). This phenomenon has been discussed in some detail by Ling (74).

Studies on the binding of ions to polyelectrolytes are of great theoretical interest, but there are many reasons why the results of these studies (the observed magnitude and selectivity of binding) are of little direct biological significance. Not the least of these reasons is the fact the proteins in a living cell are not free in solution, but more or less spatially fixed, and in a muscle fiber at least, cross-linked to a fairly high degree. Thus, it is logical to investigate the binding of the alkali metal cations to spatially fixed and cross-linked polyelectrolytes; ion exchange resins.

Ion Exchange Resins and the Alkali Metal Cations. In an ion exchange resin, two ions generally exchange with one another in stoichiometric quantities, but they are not generally held equally strongly by the exchanger (75). The stoichiometric exchange between two monovalent cations, A and B, present in both the aqueous and the exchanger phase, may be represented by the equation

$$\bar{A} + B \quad A + \bar{B} \quad \text{.....[6]}$$

where the bars denote the exchanger phase. The equilibrium selectivity coefficient, $K_{B/A}$, may then be defined as

$$K_{B/A} = (\bar{X}_B/\bar{X}_A)(X_A/X_B) \quad \text{.....[7]}$$

where $\bar{X}_A$ and $\bar{X}_B$ represent the equivalent fractions of the counterions in the exchanger and X_A and X_B represent the equivalent fractions of these ions in the solution (75). It is apparent that if both counterions are monovalent, either the molar or molal concentrations of the ions in the exchanger or the solution phase could have been used in Eqn. [7], since only the ratio of the quantities appears. If the activity coefficients of the two ions in the solution phase are different, a correction may be applied by multiplying the selectivity coefficient by the ratio of the activity coefficients, (f_A/f_B). Thus, the corrected selectivity coefficient, $K'_{B/A}$, is defined as

$$K'_{B/A} = K_{B/A}(f_A/f_B) \quad \text{.....[8]}$$

Both the order in which a resin selects the alkali metal cations and the magnitude of the selectivity depend on several factors. The most important appear to be the nature of the anionic site, the structure and degree of cross-linking of the resin and the relative concentrations of the two ions in the resin. The specific capacity of the resin (the number of exchange groups per unit amount of exchanger), the ionic strength of the surrounding solution, and the temperature may also affect the selectivity. The effect of the nature of the anionic site on the selectivity will be discussed first, and the discussion will be limited to three cations; Li, Na and K.

The magnitudes of the selectivity coefficients of monosulfonated cross-linked polystyrene resins for the above cations vary with the degree of cross-linking and the relative concentrations of the ions in the exchanger, but the order of selectivity is K>Na>Li (75). The results obtained

on carboxylic and phosphonic resins are of greater biological significance. The order, as well as the magnitude of the selectivity of a carboxylic resin depends on several factors. If, however, the resin is moderately cross-linked, maintained at a neutral or alkaline pH and the two competing ions are present in approximately the same concentrations in the resin, it will select the cations in the order Li>Na>K (76, 77). This order of selectivity was observed on carboxylic resins with three different types of polymer matrix, different specific capacities and different degrees of cross-linking (75). The importance of the anionic group in the determination of the selectivity is illustrated by the fact that no sulfonic resin is known which prefers sodium to potassium (75). Bregman and Murata (78) have shown that phosphonic resins, under alkaline and neutral conditions, also prefer the alkali metal cations in the order Li>Na>K. Below pH 6, when the resin exists mainly in the $-P(OH)O_2^-$ instead of the $-RPO_3^=$ form, the selectivity order is reversed, potassium being preferred to sodium (76, 78).

The effect of variations in the relative concentrations of the cations on the selectivity of a resin is sometimes quite pronounced. As Bregman (76) has stated, "In general, for resins of a conventional degree of cross-linking and a pair of cations which differ significantly in size, it has been found that the affinity for a cation increases as its mole fraction in the resin phase decreases". This statement is certainly applicable to the selectivity of the carboxylic and phosphonic resins for the three alkali metal cations discussed above (76, 77). The selectivity coefficient $K_{Li/K}$ for a carboxylic resin cross-linked with 15.4% divinylbenzene (DVB), for example, increases by a factor of about 5 as the mole fraction of Li in the resin decreases from .9 to .2 (76).

Variations in the percentage of cross-linking agent in a resin (usually DVB) result in less predictable changes in the selectivity than variations in the mole fraction of a cation in the resin. In sulfonic resins, increasing the percentage of cross-linking agent generally increases the selectivity of the resin (75). In carboxylic resins, increasing the percentage of cross-linking agent increases $K_{Li/K}$ but decreases $K_{Na/K}$ (77).

One of the most important variables affecting the selectivity of carboxylic and phosphonic resins appears to be the pH, or equivalently, the degree of neutralization, $\bar{\alpha}$, of the resin. A carboxylic resin with 6% DVB, for example, selects the alkali metal cations in the order Li>Na>K at pH 7.4 ($\bar{\alpha}$ = .85), but the order of selectivity is reversed at pH 6.5 ($\bar{\alpha}$ = .2) (77). Similarly, the selectivity of phosphonic resins is reversed under acid conditions (76, 78).

Bregman (76) has discussed the effects of ionic strength and temperature on the selectivity of a resin and Reichenberg (75) has commented on the effects of variations in the specific capacity. Unfortunately, little work has been done on the effect of variations in the latter parameter. Indeed, it is unfortunate, as Reichenberg (75) has stated, that "the understanding of selectivity phenomenon with monovalent ions (the alkali metal cations in this case) is of relatively little importance technologically", although it "is of the greatest importance in connection with biological phenomenon".

Its biological importance, from the point of view of this thesis, lies in the analogy between the inorganic, fixed charge, ion exchange systems and the spatially fixed proteins in the living cell. A solution of proteins extracted from a cell may be considered to be analogous to a poly-

electrolyte solution. As both the number of cations bound, and the selectivity with which the polyelectrolytes bind these cations can be altered (and in general increased) by charge fixation and cross-linking, one should not expect the binding characteristics of extracted proteins to be identical to those of proteins in the living cell. The theories discussed below indicate the importance of the structure of a protein in determining its bind-ind characteristics.

Theories of Cation Selectivity. The first "mechanistic" attempt to explain the selectivity of an ion exchange resin was made by Gregor (79, 80). The basic factor governing selectivity was assumed to be the elastic forces in the resin, which would oppose the tendency of the resin to swell. He reasoned that the cation with the smallest hydrated radius would cause the least swelling in the resin, hence be preferred in the resin phase. This led to the prediction that a resin would select the alkali metal cations in the order Cs>Rb>K>Na>Li, a selectivity order which is indeed valid for most sulfonate resins. The theory, however, now appears to be invalid because it cannot adequately explain "crossovers" or "selectivity reversals" (75, page 252).

The hydration of ions also plays an important role in a theory of selectivity developed by Eisenman and his co-workers (81-84). In this theory, however, there is no necessity to rely on the rather vague concept of the hydrated ion radius. (Kavanau (19, pages 224-248) may be consulted for a biologically oriented review of the current concepts regarding the hydration of ions.) Glueckauf (85) had criticized Gregor's theory on these grounds even before the other difficulties inherent in Gregor's theory were apparent. Eisenman's theory will first be considered in relation to a

glass (or resin) which completely excludes water.

The exchange of a cation, I, initially in combination with an anionic site, X, in the glass or resin for another cation, J, initially in a dilute solution may be represented as

$$XI + J \rightleftharpoons XJ + I + \Delta F^{o}_{ij} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots [9]$$

where ΔF^{o}_{ij} represents the standard free energy change for the process (84, see equation 8). The standard free energy change, in the ideal case, is related to the selectivity coefficient of the glass or resin by Eqn. [10]

$$\Delta F^{o}_{ij} = -RT \ln K_{I/J} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots [10]$$

where R and T have their usual significance (84, 75). The standard free energy change in the exchange process will depend mainly on two factors; the difference in the partial molal free energies of hydration of the two ions in the aqueous phase, $(\bar{F}^{hyd}_{I} - \bar{F}^{hyd}_{J})$, and the difference in the partial molal free energies of interaction of the cations with the glass, $(\bar{F}^{glass}_{I} - \bar{F}^{glass}_{J})$. Thus

$$\Delta F^{o}_{ij} = (\bar{F}^{hyd}_{I} - \bar{F}^{hyd}_{J}) - (\bar{F}^{glass}_{I} - \bar{F}^{glass}_{J}) \quad \ldots\ldots\ldots\ldots [11]$$

The values of the first term in parenthesis are known experimentally. Nothing need be known about the exact manner in which water interacts with the ions. Values are given in Eisenman's paper (84) referred to Cs. Eisenman points out that there are two independent methods of obtaining values for the second term in parenthesis of Eqn. [11]; a rather empirical, thermochemical method, and a more theoretical, atomic method. Only the latter approach will be considered here.

If the sites on the glass or resin are widely separated, the free energies of interaction between a cation and an anionic site will be given

to a first approximation by Coulomb's law for rigid sites and counterions;

$$\bar{F}_I^{glass} = -322/(r_I + r_-) \quad \text{.....[12]}$$

$$\bar{F}_J^{glass} = -322/(r_J + r_-) \quad \text{.....[13]}$$

where r_I and r_J are the naked radii of the ions I and J, and r_- is the "equivalent" radius of the anionic site (84). These two equations, along with the known values for the free energies of hydration of the cations may be substituted into Eqn. [11]. Values of ΔF^o_{ij} may then be plotted as a function of one variable, r_-. This generates a series of selectivity orders (84, see Fig. 16). If the anionic sites are very closely spaced, the free energies of interaction will be given by the following equations;

$$\bar{F}_I^{glass} = 1.56\ (-322)/(r_I + r_-) \quad \text{.....[14]}$$

$$\bar{F}_J^{glass} = 1.56\ (-322)/(r_J + r_-) \quad \text{.....[15]}$$

which are the Born-Lande expressions for the internal free energies of an alkali halide crystal lattice. The substitution of these equations instead of [12] and [13] into Eqn. [11] and the plotting of the values of ΔF^o_{ij} against r_- yields almost the same selectivity sequences (84, see Fig. 17). The value of r_- at which a given selectivity sequence is observed, however, is shifted, and it is important to note that the shift occurs in such a direction that $K_{Na/K}$ is enhanced (84, compare figures 16 and 17). For example, if the r_- of the anionic sites has a value which implies that the selectivity, $K_{Na/K}$, is unity when the sites are isolated, the value of $K_{Na/K}$ will be greater than unity of overlapping of the sites occurs.

The relationship between r_- and the selectivity may be made clearer by a consideration of two limiting cases. Consider first the case when r_- is large ($r_- \gg r_I, r_J$). This Eisenman terms a site of low field

strength. Irrespective of the relative sizes of the two cations, the second term in Eqn. [11] will be small, and ΔF^{o}_{ij} will depend primarily on the partial free energies of hydration. The glass or resin would then prefer the alkali metal cations in the order Cs>Rb>K>Na>Li (84, see right of either figure 16 or 17). Conversely, when r_- is small, that is, a site of high field strength, the second term in Eqn. [11] will predominate. The glass or resin would then prefer the cation with the smallest naked radius, that is, Li>Na>K>Rb>Cs (84, see left of either figure 16 or 17). For intermediate values of r_-, 9 other possible sequences are predicted. Thus, 11 sequences out of a possible $5! = 120$ sequences are predicted by the theory.

These theoretical predictions have been almost completely confirmed by experiments conducted on glasses of various compositions (containing sites of varying field strengths). The experimental confirmation of the theory will not be discussed here (83, 84). It need only be noted that "the general agreement between the theoretical predictions and the experimental results is sufficiently good to justify the opinion that Eisenman's theory is basically sound" (75).

The above discussion has been limited to glasses or resins which exclude water. Eisenman investigated theoretically the effect of water on the selectivity of glasses and resins by considering a water swollen resin as analogous to a concentrated solution of a strong electrolyte (83, 84). This was essentially an extension of the investigation of Cruickshank and Meares (86). Eisenman's "assumption that the free energy data of an aqueous solution may be used to represent completely the selectivity properties of an ion exchange phase containing comparable amounts of water" (83, page 313) appears debatable to this investigator, but the conclusion he derives from

the analysis is adequately supported by experimental evidence. Eisenman concludes that the main effect of water swelling will be on the magnitude of selectivity (decreasing it) and that swelling will have little effect on the pattern of selectivity. Support for this conclusion comes from experiments on both glasses and resins. The selectivity of glasses which prefer potassium to sodium passes through a maximum as the field strength of the sites is lowered, whereas the theoretical analysis indicates the selectivity should be a monotonic function of field strength. This maximum corresponds to an observable increase in the hydration of the glass, hence supports the conclusion that hydration lowers the magnitude of the selectivity (84, see right of figures 8 and 9). The data of Reichenberg (75) indicates that the selectivity of sulfonate resins is a simple function of the average amount of water per exchange group. This result also supports the conclusion that an increase in hydration leads to a decrease in the magnitude of selectivity, but does not change the order of selectivity.

Eisenman's theory permits at least a qualitative explanation of the dependence of selectivity on the nature of the anionic site. The equivalent r_- should be related to the pK_a of the ion exchange site (84). Sites with a high pK_a should have a low r_- and vice versa. Thus, it is reasonable that the sulfonic resins (the sites of which have a low pK_a, low field strength or low equivalent r_-) prefer the alkali metal cations in the order K>Na>Li. The pK_a of the sites on the other resins considered in this chapter increase in the order $-P(OH)O_2^-$, $-COO^-$, $-PO_3^=$. The magnitude of selectivity is reduced in $-P(OH)O_2^-$ resins, the order of selectivity reversed in $-COO^-$ resins (they generally prefer Li>Na>K), and the magnitude of this reversed selectivity enhanced in $-PO_3^=$ resins in agreement with the theory.

Eisenman's theory was created specifically to explain the selectivity characteristics of cation selective glasses, and has been very successful in doing this. As Reichenberg (75) has pointed out, however, detailed predictions made on the basis of this theory about the selectivity orders expected in carboxylic resins have been far less successful. The selectivity reversals in carboxylic resins, for example, commence with the sodium-lithium reversal rather than the cesium-potassium reversal, as predicted by the theory (75). This is perhaps not too surprising, because polarization and other effects have been ignored in Eisenman's theory.

Some facets of Ling's theory (74) of cation selectivity will now be discussed. The theories of Ling and Eisenman are similar in many respects, but that of the former was specifically formulated for a biological fixed charge system. Ling, like Eisenman, considered the field strength of the anionic site to be of prime importance in determining selectivity. He used a parameter he termed a "c value" (which is similar to the r_- utilized by Eisenman) to describe the field strength of the anionic site. This c value, calculated in Å, is the distance that a unit negative charge on the anion should be thought of being moved (either towards or away from the cation) to simulate the induction, multipole, polarization and direct effects of other ionic and polar groups.

In contrast to Eisenman, Ling included the possible effects of induced dipole interactions in his analysis. He also assumed that an integral number of water molecules could be found between the anion and the cation, and considered this effect statistically. The attractive forces between the anion and the cation were balanced against the repulsive forces (one of the significant latter forces being Born repulsion) and the theory

was developed in one dimension. The correct value of the polarizability of the carboxyl sites is not known, hence a number of values were considered.

It is significant that Ling's analysis predicts exactly the same 11 selectivity sequences for the 5 alkali metal cations as Eisenman's theory. This agreement, however, may be fortuitous. Many of the terms in Ling's basic equations are only rough approximations to the actual physical forces involved. The values that Ling used for the polarizability term have received special attention, for Ling (87) noted that the use of different values for the polarizability of the carboxyl groups would generate different selectivity orders. Reichenberg (75) comments that the use of higher values for the polarizability "comes closer to predicting the sequences that are found experimentally" for carboxylic resins of high specific capacity.

An equally important criticism of Ling's theory is that it predicts the entry of water into an exchanger can increase, rather than decrease the selectivity (74). Experiments performed on glasses (84) and resins (75) contradict this conclusion, as discussed above. Ling was able to make this prediction because he assumed at the start of his analysis that all the cations in a biological fixed charge system are associated with fixed anions (74), a highly dubious assumption. Ling of course, must argue in this manner to provide a theoretical justification for his contention that the living cell as a whole is analogous to an ion exchange resin with an extremely high selectivity for potassium over sodium. (According to Ling (74, page 220) the $K_{K/Na}$ for a muscle fiber must be about 300, even though he admits that no physical system is known that has a greater $K_{K/Na}$ than 10.) Further criticism of Ling's theory may be found in a paper by Conway (88).

There is no objection to considering a membrane-free biological system as an ion exchange resin (and glycerinated fibers will be considered as such below), but it does seem unreasonable, as Ling has done (74), to completely ignore the membrane surrounding a living cell. If, as Reichenberg contends, "we may to a first approximation, regard the effect of additional water as merely to 'dilute' the processes giving rise to selectivity" (75), it would seem reasonable in a living cell to consider the proteins (including that water of hydration which excludes alkali metal cations) as ion exchange particles immersed in an aqueous salt solution. This approach seems especially desirable now that accurate measurements of the activities of sodium, potassium and hydrogen in the aqueous solution surrounding the proteins can be made.

Proteins and the Alkali Metal Cations. The difficulties inherent in the application of a theory of cation selectivity to a biological macromolecule are apparent. Even if one accepted uncritically an existing theory, it would be impossible to predict whether a given protein would prefer to bind sodium or potassium, and equally difficult to predict the number of cations it would bind. It seems reasonable, however, to accept that the selectivity of a protein will depend on the r_- value (in terms of Eisenman's theory) or the c value (in terms of Ling's theory) of the carboxylic sites. It was noted that sulfonic resins (pK = 1.5) prefer to bind K>Na and that carboxylic resins (pK = 6) prefer to bind Na>K. As the pKs of the aspartic and glutamic residues of proteins lie between these two values (89), the sites could conceivably prefer to bind either sodium or potassium. It should be noted, however, that "the alkali metal cations are scarcely bound at all" to most proteins (90, page 591). Thus, if a given protein does bind significant quantities of the alkali metal cations, it may be expected that

the overlap and induction effects considered by Eisenman and Ling are of importance. Overlap effects would be expected to depend much more critically on the secondary and tertiary structure of the protein than induction effects. Some experimental studies on the binding of the alkali metal cations to extracted muscle proteins will now be discussed.

Szent-Györgyi noted the extensive binding of the alkali metal cations to extracted myosin (91). Furthermore, he noted that the ability of myosin to bind the alkali metal cations is extremely labile. The binding decreases markedly after storage of myosin at 0^{o} for only 24 hours, and is completely abolished by a thermal denaturation of the protein. Two conclusions may be made from these observations. First, they illustrate that the ability of myosin to bind cations is dependent on the secondary and tertiary structure of the molecule. Second, they indicate that the binding of cations to myosin in the living cell may be somewhat higher than that measured on the extracted protein because of denaturation during the extraction procedure.

Lewis and Saroff (92) made careful measurements of the binding of sodium and potassium to actin, myosin and actomyosin. Although actin and myosin have similar isoelectric points and amino acid compositions, it was found that actin does not bind potassium ions but that myosin binds both sodium and potassium ions quite strongly. The maximum number of alkali metal cations that could be bound to myosin was about 50 moles/10^{5} g myosin.* At a physiological pH of about 7.3 (see Chapter V) and a free

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* Unfortunately, Lewis and Saroff (92) were not as careful in describing their experiments as they were in performing them. They initially defined

potassium concentration of 0.100 M, about 35 moles of potassium are bound to 10^5g of myosin (92). Myosin binds sodium even more strongly than it binds potassium. At a pH of 7.7 and a temperature of 5° C, the apparent association constant of myosin for sodium (225 ± 22) is about twice that of myosin for potassium (98 ± 11). These "anomalously high association constants for the binding of Na and K to myosin" (92) and the fact the binding depends on the structure of the molecule imply that overlap effects should account for most of the binding. According to theory (84), the overlapping of sites should enhance $K_{Na/K}$. Thus, it is logical that sodium is bound more

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$\bar{\nu}_K$ as the "average number of potassium ions found per mole of myosin" (92, page 2115). It is apparent from figures 1 and 2 in their paper that $\bar{\nu}_K$ has a maximum value of about 50. On page 2116, however, (see Table II) the maximum number of ions bound per 10^5g of myosin is also stated to be equal to 50. Thus, it is not clear whether $4.2 \cdot 10^5$g (the approximate weight of one mole of myosin) or 10^5 grams of myosin is capable of binding about 50 moles of alkali metal cations. Cope (93), for one, initially accepted the former interpretation. A careful reading of the paper, however, indicates that the latter interpretation is correct. Lewis and Saroff mention the molecular weight of myosin only once in their paper, and then only when they discuss the combination of actin and myosin in approximately molar ratios. Furthermore, in this (92) and a later paper (94) they correlate the results directly with the fact that there are "15 histidine residues per 10^5 grams of myosin". Cope (personal communication) has since agreed that the results of Lewis and Saroff indicate that 50 moles of cations are bound per 10^5g of myosin.

strongly than potassium to myosin. (It is also logical that small molecules which can form alkali metal chelates (94) prefer these cations in the order Li>Na>K.) In a later paper, Saroff (94) analyzed the dependence of the binding on pH, and concluded that "the binding of sodium and potassium ions appears to involve carboxyl-alkali metal-imidazole and carboxyl-alkali metal-amino chelates". This conclusion is certainly reasonable, but the possibility that other pairs of sites could be involved in the binding cannot be excluded on the basis of the available evidence.

It may be of interest to calculate roughly the maximum number of binding sites in the living cell for the alkali metal cations from the data of Lewis and Saroff (92). Myosin comprises about 39-57% (95, 96) of the total protein in a muscle. It will be assumed that it comprises 50% of the protein in a barnacle muscle fiber. These fibers contain about 75% water by weight (Chapter IV). It is not unreasonable to assume that 80% of the solid material in the cell is protein. Thus, about 10% of the weight of a barnacle muscle fiber consists of myosin. At the physiological pH of 7.3 (Chapter V), about 40 sites should be available to bind sodium and potassium per 10^5 g of myosin (92). Thus, the results of Lewis and Saroff predict that about 50 mMoles of alkali metal cations/Kg of fiber water are bound to myosin in a barnacle muscle fiber.

The binding of sodium and potassium to muscle proteins in glycerinated fibers will now be considered. This system should be a better model of the living cell than dilute solutions of extracted proteins. In a glycerinated fiber the proteins are spatially fixed in such a manner that the structure of the proteins (in the thick and thin filaments) is similar to that of the proteins in a living muscle fiber. Fenn (97) equilibrated

glycerinated muscle fibers in solutions containing equal concentrations of sodium and potassium, then measured the concentrations of these cations in muscle fibers.

Two major conclusions may be drawn from his data. First, more sodium than potassium was accumulated by the muscle fibers at all the external concentrations studied, the preference for sodium over potassium being most marked at the lower concentrations. Slightly more "binding" occured than would have been predicted from the results of Lewis and Saroff (92), but Fenn cautions that "the detailed and quantitative interpretation of these figures must await further experiments" (97). The other interesting feature of the data is the fact the number of bound ions/Kg muscle appears to pass through a maximum, then decrease as the external concentrations of the ions is increased. The significance of this trend will be considered in more detail in Chapter IX, but it may be noted here that this result could be explained if a fraction of the water in the glycerol extracted muscle fiber is unavailable to act as solvent for the alkali metal cations.

One final point may be made about the binding of ions to the contractile proteins in the living cell. It would be naïve not to expect the binding of other charged species to influence the binding of the alkali metal cations to these proteins. Thus, the binding of calcium and magnesium would intuitively be expected to decrease the binding of the alkali metal cations to myosin, whereas the binding of anions such as chloride would be expected to enhance the binding of the alkali metal cations (91). Bound polyphosphates such as ATP (98, 99, 100) might be expected to act like the anionic moieties on a phosphonic ion exchange resin (76, 78) and enhance the

overall $K_{Na/K}$ of the contractile proteins, but the experiments of Fenn (97) indicate that ATP has very little effect on the binding of sodium and potassium to the contractile proteins in a glycerinated muscle fiber.

In summary, it was noted that although very few small molecules bind the alkali metal cations (strong chelating agents being the exception), these cations can engage in ion pair formation with biologically significant anionic moieties on polyelectrolytes and ion exchange resins. Carboxylic resins prefer to bind sodium to potassium ($K_{Na/K}>1$), but it was noted that there was little theoretical justification for an extrapolation of this result to the proteins in a living cell. Induction and overlapping effects (which depend on the structure of the protein) could change the anionic field strength of the sites, hence the selectivity and magnitude of the binding. Experimental studies on the binding characteristics of the major protein in a muscle fiber, myosin, indicate that this protein is unique in possessing relatively high association constants for both sodium and potassium, the former ion being bound more strongly than the latter. The critical dependence of the binding characteristics of myosin on the structure of the molecule and the preference of myosin for sodium over potassium are compatible with the existing theories of cation selectivity. As the binding characteristics of extracted myosin are qualitatively similar to those of the proteins in a glycerinated muscle fiber, it seems reasonable to expect that significant quantities of both sodium and potassium will be bound to myosin in the living cell, and that the selectivity of the protein, $K_{Na/K}$, will be greater than unity.

CHAPTER III

SCOPE AND PURPOSE OF THE INVESTIGATION

The main purpose of this investigation was to test experimentally the hypotheses that: i. a significant fraction of the alkali metal cations in a striated muscle fiber is bound to myosin,

ii. the binding sites on the thick filaments in a striated muscle fiber prefer to bind the alkali metal cations in the order $Li > Na > K$,

iii. some of the water in a striated muscle fiber is "bound" in such a manner as to be unavailable to act as solvent for the alkali metal cations free in the myoplasm. Four separate experimental approaches, which are briefly outlined below, were adopted.

Chapter IV. The hypotheses were first tested by comparing the activities of sodium and potassium in the myoplasm to the values expected from a determination of the total sodium and potassium content of the cell. The activities were measured directly by means of cation sensitive glass microelectrodes and the total content of sodium and potassium in the same muscle fiber was determined by a conventional flame photometric analytic technique. It is apparent that if proportionally more sodium than water is "bound", the measured activity of sodium will be less than the activity predicted from the analytic measurements. It is also apparent that if proportionally more water than potassium is "bound", the measured activity of potassium will be greater than the activity of potassium predicted from the analytic measurements. The results obtained are compatible with both of these predictions.

There were other reasons for performing the above experiment. A knowledge of the activities of sodium and potassium in the myoplasm is a prerequisite to an accurate measurement of the membrane permeabilities and transport characteristics of these ions. A proper evaluation of the membrane potential of the cell, and the intracellular reactions (ATPase activation, for example) these cations can undergo also depends on an accurate knowledge of the intracellular activities. Finally, the electrode measurements provided direct experimental evidence which contradicted Ling's hypothesis (1) that potassium is accumulated preferentially over sodium by muscle fibers because it is selectively adsorbed on intracellular binding sites.

The microelectrode measurements were compatible with, but did not prove the hypothesis that a significant fraction of sodium in the cell was bound to myosin. All or part of the sodium in the cell unavailable to the cation sensitive microelectrode could have been sequestered in intracellular organelles. The next experiment was designed specifically to test the hypothesis that at least some of the "bound" sodium in the cell was indeed bound to myosin.

Chapter V. It is known that extracted myosin undergoes thermal denaturation at 37° C (2) and that this denaturation causes the release of bound alkali metal cations (2) and polyphosphate anions (3). It was reasoned that if a significant fraction of the intracellular sodium was bound to myosin, a release of bound sodium, hence an increase in the activity of sodium in the myoplasm, would occur when the muscle fiber was heated to 37° C. This prediction was confirmed by measuring the activity of sodium in the myoplasm of striated muscle fibers by means of a cation sensitive glass microelectrode while the temperature of the sodium free bathing

solution was raised to 37° C. The experiments reported in Chapter V provided strong evidence that much of the sodium in striated muscle fibers was indeed bound to myosin, but it was thought desirable to obtain experimental evidence independent of microelectrode measurements to substantiate this conclusion.

Chapter VI. A prediction can be made about the light scattering characteristics of a striated muscle fiber on the basis of the hypothesis that sodium is bound to the thick filaments. The turbidity or optical density of a solution of macromolecules is intimately related to the net charge on the macromolecules. If the net charge is increased, the turbidity of the solution decreases (4, 5, 6). Thus, the turbidity of a muscle fiber would be expected to decrease if the net charge on the main scattering centers in the fiber, the thick filaments, was increased. Bathing the fiber in a sodium free solution should cause sodium to move off the binding sites on the thick filaments and out of the cell. If no ion replaces sodium on the binding sites, the net negative charge on the thick filaments should increase, and the turbidity of the fiber should decrease. This prediction was confirmed for fibers bathed in sodium free solutions containing sucrose, tris or potassium as substitutes for sodium. When lithium was used as a substitute for sodium in the bathing solution, the turbidity of the fibers increased slightly. This finding is also compatible with the working hypothesis, for the lithium entering the cell should be bound more strongly than sodium to the sites on the thick filaments (7).

Chapter VII. The last series of experiments was designed to measure the selectivity, $K_{Na/K}$, of the proteins in a glycerinated fiber when the free concentrations of sodium, potassium and hydrogen were similar to

those found in the myoplasm of a living cell. The concentrations of sodium and potassium accumulated by the glycerinated fibers were measured by means of radioisotopes and a standard flame photometric technique. The results indicated that the selectivity of the proteins, $K_{Na/K}$, was indeed greater than unity, but also that the number of sodium ions bound to the proteins was not great enough to explain the extremely low activity of sodium in the myoplasm of a living fiber. Thus, it was concluded that in a living muscle fiber either some factor enhances the binding of sodium to the contractile proteins or sodium is compartmentalized in intracellular organelles.

CHAPTER IV

ACTIVITY OF SODIUM AND POTASSIUM IN THE MYOPLASM

A. Introduction

The motivation for measuring the activities of sodium and potassium in the myoplasm of striated muscle fibers was discussed in the previous two chapters. A means of measuring these activities was devised by Hinke (1), who was the first to construct cation sensitive glass microelectrodes from the glasses developed by Eisenman and his coworkers (2). The success of the experiments also depended on the use of the extremely large (typical weight = 20 mg, typical diameter = 1mm, typical length = 4 cm) muscle fibers of the giant barnacle, Balanus nubilus. Hoyle and Smyth (3) may be consulted for a description of the barnacle muscles.

One advantage of performing experiments on the large barnacle muscle fibers is that relatively large microelectrodes can be used. These electrodes are easier to construct than the extremely small cation sensitive microelectrodes that Lev (4, 5) utilized for measurements on frog muscle fibers. Another advantage is that the muscle fibers can be dissected without damage because they are held together with only a loose network of collagen. The tendon can be cannulated without damage to the fiber and the microelectrode inserted down the longitudinal axis of the fiber; a procedure which ensures that the cation sensitive tip is far from the region of damaged membrane. If the microelectrode is inserted transversely, the tip is near a damaged area of membrane, and leakage of sodium into the fiber, or of potassium out of the fiber can occur. Finally, the total sodium and potassium content of a single barnacle muscle fiber can be accurately

determined by means of flame photometry because of the large size of the fibers.

B. Methods

Microelectrodes. The sodium sensitive microelectrodes were constructed from Corning NAS 11-18 (sodium sensitive) and 0120 (lead) glasses by a method first described by Hinke (1). The joint between the outer lead glass shank and the sodium sensitive tip was formed by fusing the two glasses in a microforge. The sensitive tips of the sodium (and potassium) microelectrodes were about 15μ in diameter and 150-300μ in length, as shown in Fig. 1. A recent article by Hinke (6) contains details of the

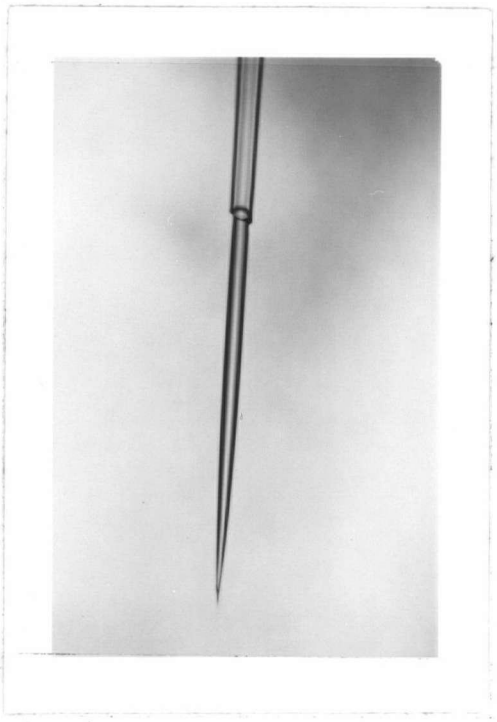

Fig. 1. Photograph of the tip of a sodium sensitive microelectrode. The tip is about 15μ in diameter and 300μ in length.

construction procedure. The potassium sensitive microelectrodes were constructed from Corning NAS 27-3 (potassium sensitive) and 0120 (lead) glasses. Only a few microelectrodes were constructed by the method

described by Hinke (1, 6), which involved matching and fusing three sets of glasses (NAS 27-3, 0120 and pyrex) at a joint. A new technique was developed by the author whereby a joint was formed by melting beeswax between the outer lead glass shank and the inner potassium glass capillary. This method of construction requires much less skill than the previous technique and the electrodes have slightly superior characteristics (a longer life and better selectivity, presumably because the glass is heated less). A description of this technique will appear in an article by Hinke (7).

The equations which describe the behavior of the sodium and potassium sensitive microelectrodes are

$$E_{Na} = E'_{Na} + S_{Na}\log_{10}(a_{Na} + k_{Na}a_K) \quad \text{.....[16]}$$

$$E_K = E'_K + S_K\log_{10}(a_K + k_K a_{Na}) \quad \text{.....[17]}$$

where E_{Na} and E_K are the measured potentials (millivolts) of the microelectrodes in solutions containing Na and K ions at activities a_{Na} and a_K respectively; the other terms are constant for a given electrode and are obtained by calibration in the standard solutions.

The sodium (potassium) sensitive microelectrodes were calibrated in the following five standard solutions, which were buffered to either pH 7 or pH 8 with 0.01 M tris: 0.1 M NaCl (0.1 M KCl), 0.01 M NaCl (0.01 M KCl), 0.05 M KCl plus 0.05 M NaCl, 0.20 M KCl plus 0.05 M NaCl, and 0.40 M KCl plus 0.05 M NaCl. The potentials from a microelectrode immersed in a standard solution of either pH 7 or pH 8 were identical (±0.5 mV). Electrodes were calibrated before and after each experimental reading, and the results were rejected if the two calibrations did not coincide (± 1 mV).

The cation selectivity, k_{Na} or k_K, remained relatively constant

from day to day for a given microelectrode, but varied from electrode to electrode. For the sodium microelectrodes, the selectivity, k_{Na}, varied from 1/50 to 1/1000; for the potassium microelectrodes, the selectivity, k_K, varied from 1/1 to 1/2. The imperfect selectivity of the electrodes necessitated measuring E_{Na} and E_K on the same fiber, then solving the two simultaneous equations, [16] and [17] for the activities. The response of the sodium microelectrodes to a 10-fold change in the sodium activity, S_{Na}, was always 59 mV, whereas the response of the potassium microelectrodes to a 10-fold change in the potassium activity, S_K, was 50-55 mV. The potentials from the sodium and potassium sensitive microelectrodes were measured on a Vibron 33B electrometer and recorded on a Grass ink writing oscillograph. All electrodes used in these experiments responded correctly (±1%) to an applied potential, which indicated that the impedance of the electrodes was less than 1/100 of the impedance of the electrometer (input resistance of Vibron 33B = 10^{13} ohms).

Membrane potential measurements were made with open tip microelectrodes of the Ling and Gerard type. Only those microelectrodes with a tip potential of less than 5 mV were used, in accordance with the criterion of Adrian (8). In addition, each microelectrode was tested to ensure the potential reading in the Ringer solution and 0.40 M KCl plus 0.05 M NaCl standard solution was identical. Finally, membrane potential measurements were routinely made with two different open tip microelectrodes on the same fiber.

The potential from the open tip microelectrode was recorded via a cathode follower and a Grass P6 DC amplifier on a Grass ink writing oscillograph. Both this amplifier and the Vibron electrometer were cali-

brated before each experiment with a variable voltage source, which in turn was calibrated from a Standard Weston Cell.

Determination of Cation Activity and Concentration. Single striated muscle fibers from the depressor muscles of the giant barnacle were dissected free with a small piece of baseplate at the origin and a tendon at the insertion. A glass cannula was inserted into the tendon, but not through the muscle-tendon junction. After the cannula was ligated in position, the preparation was suspended vertically in an artificial bathing solution (Table I) as shown in Fig. 2. Cation sensitive microelectrodes

TABLE I

Solutions (mM)

	Barnacle Ringer*	Sucrose Ringer**
Na	450	
K	8	8
Ca	20	20
Mg	10	10
Cl	518	68
Tris	25	25
Sucrose		649

*pH = 7.6 for both solutions. Note the substitution of Tris for HCO_3 in the original barnacle Ringer solution of Hoyle and Smith (3).

**Sucrose added to make solution isosmotic with barnacle Ringer solution.

were manipulated through the cannulated tendon into the myoplasm until the sensitive tip was 1-2 cm from the puncture zone (See Fig. 3). This technique ensured that undamaged membrane surrounded the sensitive tip. The membrane

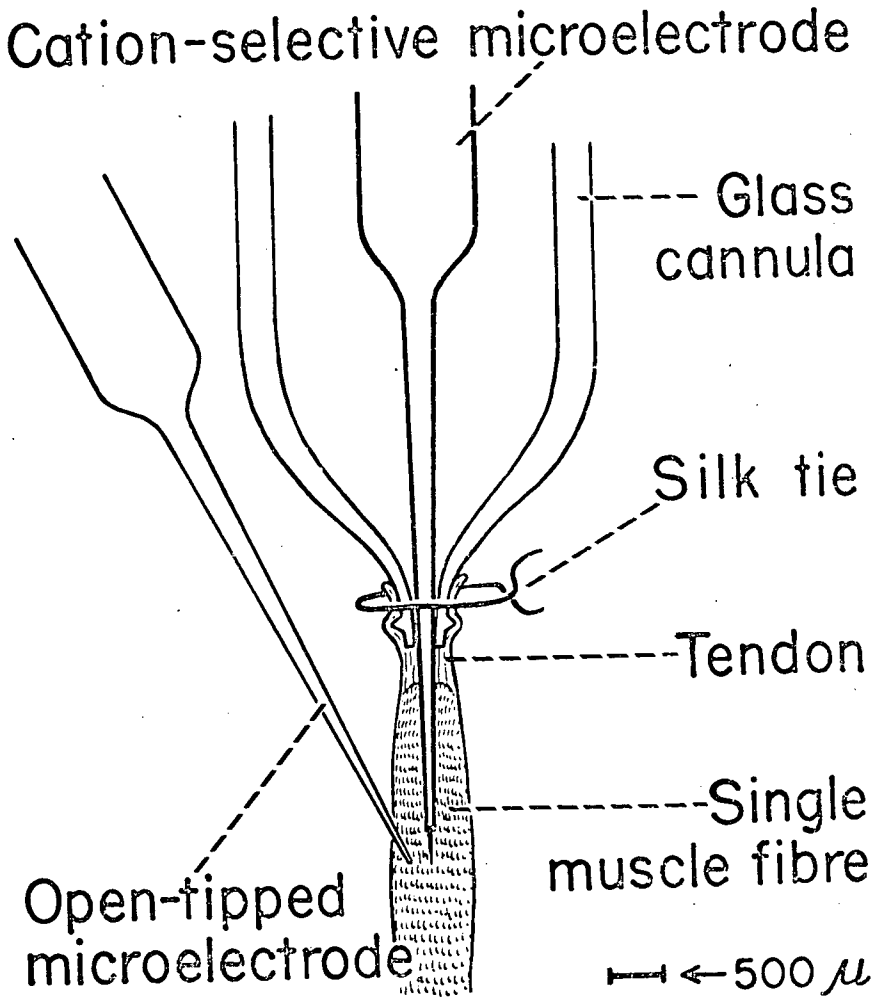

Fig. 2. Diagram of a cannulated muscle fiber with inserted microelectrode. Note the cannula does not damage the fiber membrane. See text for explanation.

potential was always measured immediately adjacent to the electrode tip, and it was subtracted from the potential of the cation sensitive microelectrode. (The cation sensitive microelectrode registers of course the membrane potential as well as the potential due to the alkali metal cations.) The same external reference electrode was used for both the experiment and the calibrations. Fibers were examined for damage spots before and after an electrode impalement. If any damage was observed, the fiber was rejected. After completion of the electrode measurements, the fiber was transferred to a Petri dish, carefully decannulated to avoid damage, and swirled for 15 seconds in isosmotic sucrose. The fiber was then blotted and placed in a weighing bottle. After drying, and digestion with nitric acid, the fiber

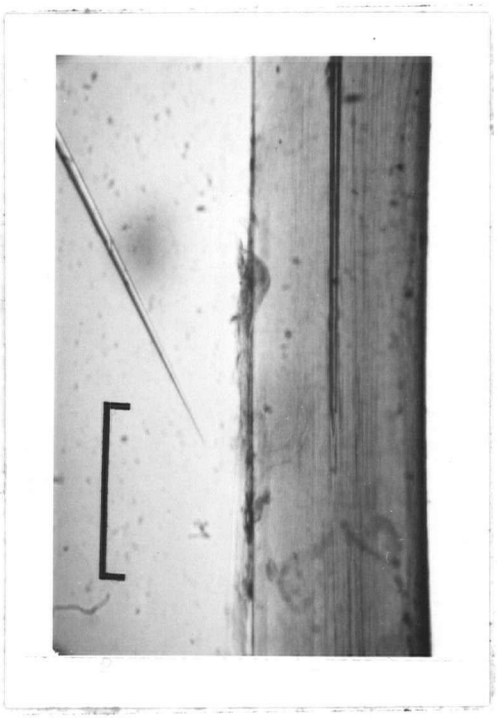

Fig. 3. Photograph of a single striated muscle fiber from the giant barnacle. The fiber was suspended vertically by ligating its tendon to a glass cannula (not shown). The cation sensitive microelectrode was inserted into the fiber via the cannulated tendon. Note a cation sensitive microelectrode in the fiber and an open tip microelectrode in the bathing solution. The vertical bar represents 1 mm.

was analyzed for total sodium and potassium content with a Unicam SP 900 flame spectrophotometer.

Determination of Membrane Potential at High External Potassium Concentration. Single muscle fibers were dissected and suspended by their tendons in the vertical plane. Eight bathing solutions with potassium concentrations ranging from 0 to 450 mM were used. The product of the potassium and chloride concentrations in each solution containing potassium was kept constant by replacing chloride with methanesulfonate. Magnesium, calcium and tris were present at the same concentrations as in barnacle Ringer solution. To eliminate spurious results due to the development of transitory tip potentials on microelectrodes within the myoplasm, membrane potential measurements were made at each potassium concentration with at least two different microelectrodes. The total potassium content of the muscle fiber was determined in the manner described in the previous paragraph.

Determination of Bound Sodium and Water. The separation of the sodium, potassium and water content of a single muscle fiber into a "bound" and "free" fraction is expressed by the following equations

$$C_{Na}V = (a_{Na}/\gamma_{Na})a_{Na}V + B_{Na} \quad \text{.....[18]}$$

$$C_{K}V = (a_{K}/\gamma_{K})a_{K}V + B_{K} \quad \text{.....[19]}$$

where γ_{Na} and γ_{K} are the molal activity coefficients of sodium and potassium in the myoplasm; V is the total water content (kilograms); α_{Na} and α_{K} are the fractions of water "free", or more specifically, available to act as solvent for the sodium and potassium ions respectively; C_{Na} and C_{K} are the concentrations of sodium and potassium (moles per kilogram fiber water)

determined by flame photometry; a_{Na} and a_K are the activities of sodium and potassium determined directly by the microelectrodes; B_{Na} and B_K are the "bound" quantities of the cations (moles). It should be stressed that any ion unavailable to affect the cation sensitive microelectrode is considered "bound". Thus, the ions compartmentalized in intracellular organelles or "trapped" in the electrostatic field of a negatively charged macromolecule, as well as those ions complexed by specific sites in the myoplasm are considered to be "bound".

These two equations, which are valid by definition, contain six quantities which cannot be determined experimentally at present: α_{Na}, α_K, γ_{Na}, γ_K, B_{Na} and B_K. Four more equations are required. These following equations are based on assumptions, and may be incorrect. The first assumption is that the activity coefficients of sodium and potassium ions free in the myoplasm are equal

$$\gamma_{Na} = \gamma_K \equiv \gamma \quad \text{.....[20]}$$

This assumption is not based on theoretical grounds. As Robinson and Stokes (9, page 454) state "The various physiological fluids can be quoted as another example where a theory of mixed electrolyte solutions would lead to progress..". It seems a reasonable assumption, if only because the activity coefficients of a 0.2 M KCl and NaCl solution differ by merely 3%. (One could, as Lev (5) has done, merely define the activity coefficients of sodium and potassium in a muscle to be the ratios of the measured activities to the measured concentrations. One is then left with the problem of explaining why the activity coefficient of sodium, defined in this manner, differs markedly from the activity coefficient of potassium in the myoplasm.)

The second assumption is that the activity coefficient of the myoplasm is equal to the activity coefficient of the barnacle Ringer solution.

$$\gamma = 0.65 \quad \text{.....[21]}$$

It should be noted that the sum of the concentrations of sodium and potassium in a barnacle muscle is, on the average, only about half the sum of the concentrations of these ions in the bathing solution (Table I and Fig. 4). Thus, the activity coefficient could be as high as that of a 0.25M KCl solution ($\gamma = 0.7$). It is probably somewhat lower because of the many charged groups on proteins in the myoplasm. Furthermore, the binding of a fraction of the intracellular water will increase the free cation concentration, and lower the activity coefficient. It seems unlikely, however, that these factors could lower the value of the activity coefficient to below 0.6 (the activity coefficient of a 1.0 M KCl solution). Thus, the assumption that the activity coefficient of the myoplasm is equal to 0.65 is only a guess, but it is probable that the actual value lies between 0.6 and 0.7. An error in the estimate of the activity coefficient of less than ± 0.05 would alter quantitatively, but not qualitatively the conclusions of this chapter. The possibility that the macroscopic dielectric constant of the muscle fiber affects the activity coefficient will be considered in the Discussion.

The next assumption is that the fraction of water available to act as solvent for sodium in the myoplasm equals the fraction of water available to act as solvent for potassium.

$$\alpha_{Na} = \alpha_K \equiv \alpha \quad \text{.....[22]}$$

This assumption may not be valid. Sodium does not fit as well as potassium

into the normal Ice I lattice, hence may be excluded from a larger fraction of water in the cell than potassium (10). The use of an overestimated value for α_{Na} in Eqn. [18], however, will merely cause the value of B_{Na} to be underestimated. For this reason, the assumption is acceptable. The final assumption is that there is no binding of potassium.

$$B_K = 0.0 \quad \text{.....[23]}$$

This assumption is almost certainly incorrect, but it was made for mathematical, not physical reasons. This assumption serves to maximize the value of $\alpha_K = \alpha$, which is calculated from Eqn. [19]. (In other words, it is the minimal fraction of "bound" water that is calculated.)

When Eqns. [20-23] are substituted into Eqns. [18] and [19], the following equations result.

$$C_{Na}V = (\alpha/0.65)a_{Na}V + B_{Na} \quad \text{.....[24]}$$

$$C_K = (\alpha/0.65)a_K \quad \text{.....[25]}$$

From these two equations and the experimental data, the minimal fraction of "bound" water and the minimal fraction of "bound" sodium in a barnacle muscle fiber may be calculated.

C. Results

Concentration and Activity Measurements. Consider now the results obtained from the concentration measurements made on the experimental and control fibers. These are illustrated in Fig. 4. It is apparent that there is a wide variation of C_{Na} and C_K in muscle fibers from different barnacles, as well as a close correlation between the C_{Na} and C_K of individual fibers (correlation coefficient = 0.95). Muscle fibers from the same barnacle showed little variation in either C_{Na} or C_K. This is illustrated in Table IIa, which

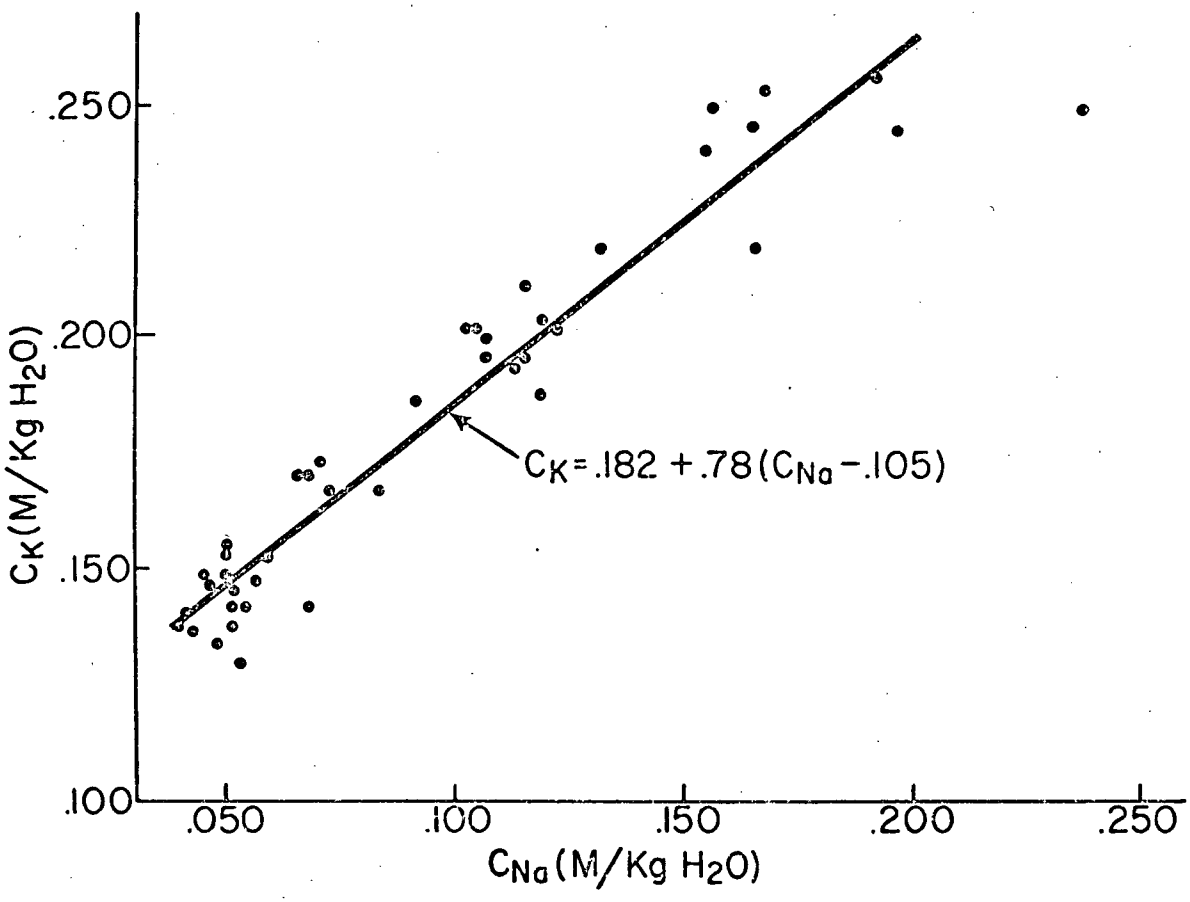

Fig. 4. Relation between the sodium and potassium contents of single muscle fibers. The correlation coefficient is 0.95.

gives the activity, concentration and membrane potential measurements made on fibers from a single barnacle. The salient features of Table IIa are that C_{Na} is much larger than a_{Na} and that C_K is approximately equal to a_K. These two features are common to all the fibers investigated.

The fractions of "bound" water and "bound" sodium were calculated for each individual fiber. That is, the values of a_{Na}, a_K, C_{Na} and C_K obtained from each experimental fiber were used to solve Eqns. [24] and [25] for $(1-\alpha)$ and $(B_{Na}/V)/C_{Na}$. By this method of analysis, the fractions of

TABLE II

Sodium and potassium in single muscle fibers*

a_K	C_K (moles/kg H_2O)	a_{Na}	C_{Na}	Membrane potential (mV)	Water content (%)
(a) Barnacle Ringer solution					
0.157 ±0.006† (3)	0.143 ±0.001 (17)	0.010 ±0.001 (3)	0.051 ±0.002 (17)	67.0 ±0.2 (3)	79.4 ±0.1 (17)
(b) Sucrose Ringer solution					
0.170 ±0.005 (8)	0.174 ±0.002 (16)	0.007 ±0.001 (8)	0.039 ±0.003 (16)	61.8 ±0.5 (8)	72.8 ±0.2 (14)
Barnacle Ringer controls					
	0.158 ±0.004 (8)		0.056 ±0.006 (8)	70.7 ±0.4 (8)	75.8 ±0.7 (8)

*All experiments were done at 25°C
†± S.E.
NOTE: Table IIb shows average results. Table IIa shows only the results obtained from the muscle fibers of a single barnacle. The C_{Na} and C_K of all the fibers in this series are shown in Fig. 4. Table IIa is presented only to indicate that there is no substantial variation in the values of C_{Na} and C_K obtained from the muscle fibers of a single barnacle. The number of determinations is shown in parentheses.

"bound" water and "bound" sodium were found to be 0.41 ± 0.014* and 0.84 ± 0.001* respectively (nine experiments). These results are similar to the values obtained by Hinke in a series of preliminary experiments, which were also reported in a paper by McLaughlin and Hinke (11). Hinke concluded from his measurements that the fractions of "bound" water and sodium were .43 and .85 respectively. The small standard error for the fraction of bound sodium is worthy of note. It indicates that this quantity remained constant even though the sodium content of the fibers varied from 0.040 to 0.200 moles/kg H_2O (Fig. 4).

Table IIb illustrates the average results obtained from fibers

*± S.E.

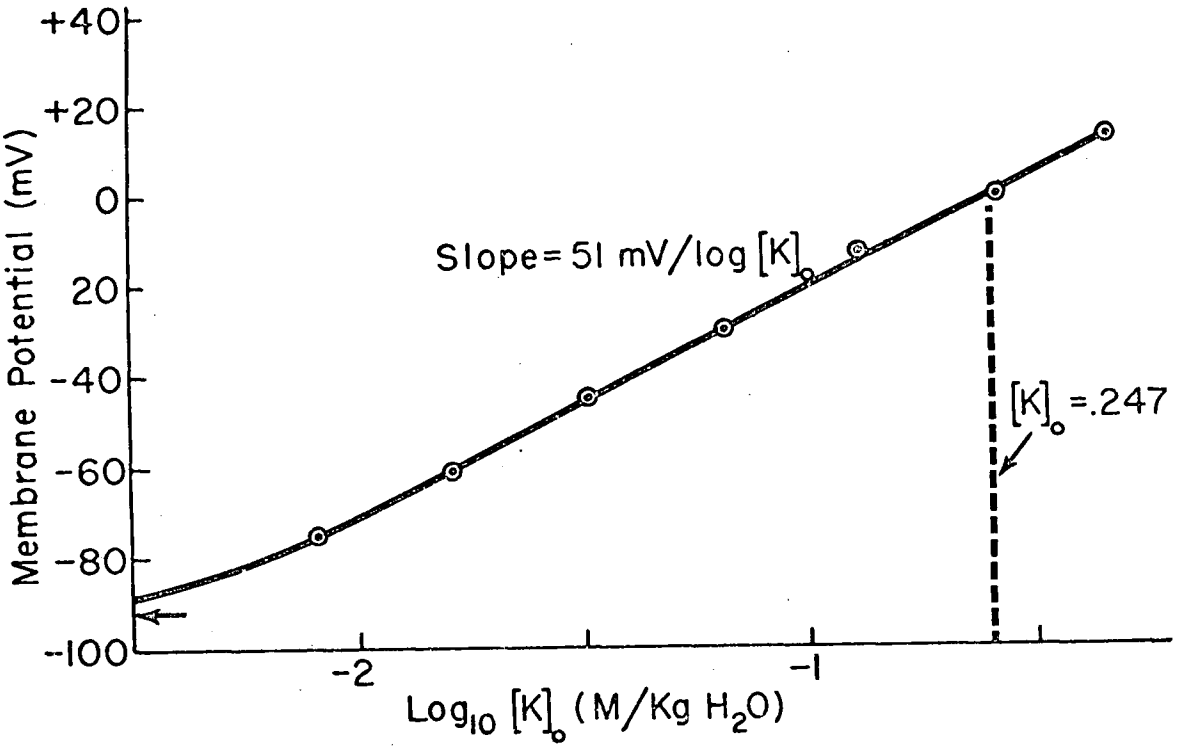

Fig. 5. Relation between membrane potential and log $[K]_o$ for a typical muscle fiber. The product $[K]_o[Cl]_o$ was maintained a constant.

bathed for 45 minutes in sodium free, sucrose substituted Ringer. When these results were used to solve Eqns. [24] and [25] for the fractions of "bound" water and sodium, the values obtained were similar to those obtained from fibers bathed in normal Ringer. The fractions of "bound" water and sodium were found to be 0.34 and 0.81 respectively.

Membrane Potential Study. The relationship between the membrane potential and the log of the external potassium concentration is shown for a typical barnacle muscle fiber in Fig. 5. The observed linear relationship

when $[K]_o$ is greater than 0.016 M and the slope of 51 mV/log $[K]_o$ indicate that the sarcolemma may have been acting as a semipermeable membrane to the potassium ion. (A linear relationship was observed for each of the five experimental fibers. The average slope was 53 ± 1 mV/log $[K]_o$.) If the potassium concentration gradient is the sole determinant of the membrane potential at regions of high external potassium concentration (admittedly a questionable assumption, see Chapter V), the internal and external activities of potassium should be identical when the membrane potential is zero. The average value of $[K]_o$ when the membrane potential was zero was 0.242 ± 0.012 moles/kg H_2O (five experiments). This corresponds to an activity of 0.242(0.65) = 0.157, which is assumed to equal the activity of potassium in the myoplasm. If this is used with the average value of C_K, 0.177 ± 0.006 moles/kg H_2O (15 experiments), to solve for α from Eqn. [25] the fraction of "bound" water is found to be 0.27.

D. Discussion

Two major criticisms of the intracellular use of cation sensitive microelectrodes have been advanced by Ling (12). His first criticism is that the proteinaceous fixed charge network in the immediate vicinity of the microelectrode may be damaged by the microelectrode, hence have its binding characteristics altered. It is apparent from Fig. 3 that the cation sensitive glass microelectrodes used in these experiments were much smaller than the muscle fibers. A comparison of the alkali metal cation concentrations and membrane potentials of the control and experimental fibers indicated that no gross damage was suffered by the cells on impalement by the microelectrodes. If a change in the binding characteristics of the proteins in the immediate vicinity of the microelectrode did occur, sodium and potassium

ions would merely diffuse down the concentration gradients set up in the myoplasm. Any anomalous concentrations of these cations close to the microelectrode would be dissipated in time throughout the cell, and the error introduced would be expected to be negligible.

Ling's second criticism (12) is that the electrodes may respond to other ions besides sodium and potassium and that they may be poisoned by high concentrations of amino acids or proteins. Hinke (7) recently investigated this possibility. He studied the effect of high concentrations of ammonium, lysine, arginine and albumin molecules on the response of sodium and potassium sensitive microelectrodes. These molecules did affect the electrodes, particularly the potassium sensitive microelectrodes, but the magnitude of the effect was very small. In a solution containing potassium ions at a concentration of 0.253 M and NH_4, lysine or arginine at a concentration of 0.05 M, the potassium microelectrode predicted a falsely high potassium concentration of 2.1, 1.2 and 0.0% respectively. In a solution containing potassium at the above concentration and albumin at a concentration of 20 gram %, the potassium microelectrode predicted a falsely high potassium concentration of about 3%. Thus, it seems unlikely that cation sensitive glass microelectrodes are greatly affected by either proteins or amino acids in the cytoplasm of living cells.

Robinson (13) has noted that proteins and amino acids have large dielectric increments (of the order of 0.1-1.0 units of dielectric constant per gram/liter) and speculated that "the dielectric constants inside cells may therefore be very considerably greater than those of the relatively protein-free solutions outside, with a consequent increase in the activity coefficients of intracellular ions". Three criticisms may be made of this

statement. First, although amino acids would increase the dielectric constant of a cell, proteins may have the reverse effect if they are spatially fixed. Schwan (14) should be consulted for an excellent review of the dielectric constants of living cells. Next, there is little evidence to support the contention that an increase in the macroscopic dielectric constant of a solution would result in an increase in the activity coefficients of the ions in the solution. (Presumably, the activity coefficient of the ion is referred to unity at infinite dilution of the ion in water.) There is a simple relationship between the activity coefficients of ions and the dielectric constants of solutions containing molecules like alcohols, which lower the dielectric constant. The activity coefficients of ions in these solutions are markedly raised (9), not lowered, as one would anticipate from Robinson's statement. Some form of the Debye-Hückel equation would admittedly predict a lowering of the activity coefficients when the dielectric constant is lowered (and vice versa), but this effect, the "secondary medium effect" is greatly overshadowed by the "primary medium effect", as discussed by Robinson and Stokes (9, pages 351-357). Finally, it should be stressed that there is <u>not</u> a simple relationship between the dielectric constant and the activity coefficients of ions in solutions of proteins and amino acids. Glycine, for example, causes a marked increase in the macroscopic dielectric constant of an aqueous solution (22.6 units per mole) but has only a small effect on the activity coefficient of NaCl (15, 16). It may either enhance or decrease the activity coefficient of this salt, depending on the relative and total concentrations of the salt and amino acid (16). Edsall and Wyman (17, Chapters 5, 6) may be consulted for a discussion of some of the theoretical approaches to the problem of the effect of proteins and amino acids on the activity coefficients of salts.

At present, one can only state that the available experimental evidence indicates that although the concentrations of amino acids and proteins found in a barnacle muscle may significantly affect the macroscopic dielectric constant of the cell, this change should not greatly affect the activity coefficients of the ions in the cell (16, 7).

Bound Water. Many investigators have attempted to measure the fraction of "bound water" in biological tissues. Before some of these results are considered, it should again be stressed that the water in a cell probably does not exist in simple bound and free fractions and that different techniques measure different properties of the intracellular water. Studies on the swelling of muscle fibers in hypotonic solutions such as Overton (18) performed can yield little information about the state of water in the cell. The sarcolemma is now known to be permeable to sodium, potassium and other solutes, and a change in the osmolarity of the bathing solution will produce a different steady state distribution of these solutes. This difficulty was circumvented by Hill (19), who soaked a muscle in an approximately equal volume of twice normal strength Ringer solution and determined the vapor pressure of the bathing solution after equilibration. If his experimental data are corrected for a numerical error (11) they predict that about 27% of the water in a muscle fiber is "bound".

Experiments have been performed on striated muscle to determine the fraction of cellular water available to act as solvent for urea (19). The results predict a negative fraction of "bound water". This obviously incorrect result may be due to the similarities between the urea and the water molecules; both are small and are capable of forming hydrogen bonds. A similar technique has been used by Bozler and Lavine (20) to demonstrate

that only about 20% of the cellular water in smooth muscle is available to act as solvent for fructose and sucrose. The argument that these sugars do not enter the fiber (21) has been countered in a recent paper by Bozler (22).

A nuclear magnetic resonance technique has been used to study the state of water in muscle (23) and nerve (24). Bratton et al (23) interpreted their results to mean that only about 0.15% of the water in muscle was "bound" whereas Chapman and McLauchlan (24) concluded that "the bulk of water inside the nerve is in a partially oriented state". This technique should prove extremely valuable in the near future for investigating the state of water in living cells, but at the present time there are many difficulties in interpreting the results of such studies (25, pages 171-248). Attempts have been made to calculate the fraction of "bound water" in muscle by freezing (26) and microwave (27) techniques, but the results obtained from these experiments may also be interpreted in a variety of ways.

A technique has recently been borrowed from colloid chemistry by Scheuplein and Morgan (28) to measure the fraction of "bound water" in keratin membranes. They measured the rate of desorption from a hydrated tissue by means of a microbalance technique. Their results indicate that the amount of "bound water" in fully hydrated stratum corneum can be as much as five times the dry weight of the tissue. The results are interesting, but the relationship between the "bound water" calculated in this manner and the water unavailable to act as solvent for solutes is unknown at present.

It is obviously important to know the fraction of water in a muscle fiber unavailable to act as solvent for the main intracellular cation, potassium. It was this fraction that was determined directly at

25° C in a normal bathing solution by the use of cation sensitive microelectrodes. The results indicate that at least 42 ± 1.4% of the water in the myoplasm is "bound" in such a manner that it is unavailable to act as solvent for the potassium ions. When the fibers were bathed in sucrose Ringer for 45 minutes the fraction of "bound water" in the cell decreased to 34 ± 3.6%. This decrease might reflect a true change in the state of water in the cell, but it could also be due to statistical variations, an increase in the activity coefficient of the myoplasm or an increase in the binding of potassium. Of these four possibilities, the last seems most likely to be true because the fibers did accumulate potassium in the sodium free sucrose Ringer solution. The results of the membrane potential experiments indicate that 27% of the water in the cell is "bound". This value, however, should be regarded only as a qualitative indication, by a method independent of microelectrode measurements, that water is "bound" in muscle fibers from the giant barnacle.

Bound Sodium. It was found that at least 84% of the sodium in muscle fibers from the giant barnacle was excluded from the myoplasm which surrounded the microelectrode. This is qualitatively compatible with the value of 70% that Lev (5) obtained from similar measurements on frog muscle, and agrees with Robertson's (29) observation that 82% of the sodium in lobster muscle could not be extruded by subjecting the muscle to pressure. These results are thus in accord with the hypothesis that significant amounts of sodium are bound to myosin in intact striated muscle fibers. The measurements do not of course prove the hypothesis, for similar results would be expected if sodium was compartmentalized in intracellular organelles rather than bound to proteins.

Nuclear magnetic resonance (NMR) measurements may prove useful in distinguishing between these two possibilities. It has been known for several years that the NMR spectrum of sodium is broadened when sodium is complexed to polyanions (30-33). The broadening is presumably due to the orientation of the sodium nucleus with respect to the nucleus of a neighbouring atom. This, in turn, could be due to the polarization of the outer shell electrons by the proximity of a charge (an ionic bond), or the addition of electrons to the outer orbitals (a covalent bond). Cope (34, 35) performed experiments on frog muscles and concluded that 70% of the sodium did not contribute to the NMR spectrum. As the compartmentalization of sodium in organelles should not broaden the NMR spectrum, the result would seem to imply that 70% of the sodium in frog striated muscle is bound to macromolecules. Unfortunately, ion pair formation is not the only factor which can cause broadening of the NMR spectrum of sodium. For example, it broadens in alcohol water mixtures as the volume fraction of alcohol increases, and becomes invisible in 95% or absolute alcohols (33). The activity of sodium in alcoholic solutions is certainly not lowered because of extensive ion pair formation. Indeed, as mentioned earlier in this Chapter, the activities of ions in general (9, page 355) and sodium in particular (preliminary experiments) increase markedly in alcoholic solutions. The broadening of the NMR spectrum in alcoholic solutions requires a proper theoretical explanation. Also, the possibility that the "nuclear spin resonance adsorption of sodium may be altered by proteins" (31) indicates that a series of control experiments on concentrated solutions of proteins which are known not to bind sodium should be made. Thus, at the present time, Cope's NMR experiments cannot be accepted as definitive evidence that there is no compartmentalization of sodium in striated muscle fibers.

Sodium will of course be contained in compartments which are formed by invaginations of the sarcolemma. Estimates of the volume of these compartments will be discussed in Chapter VI. It need only be noted here that most of the sodium in these compartments would be expected to diffuse out within 45 minutes if the fiber is exposed to a sodium free solution. The fraction of "bound sodium" in fibers bathed for 45 minutes in sucrose Ringer (0.81) is approximately the same as that of fibers bathed in normal Ringer (0.84). Thus, it may safely be concluded that most of the sodium inside the sarcolemma of a striated muscle fiber is either bound to macromolecules or compartmentalized in intracellular organelles such as nuclei, mitochondria or cisternae.

CHAPTER V

RELEASE OF BOUND SODIUM

A. Introduction

The results presented in the previous chapter indicate that over 80% of the sodium in striated muscle fibers from the giant barnacle is not free in the myoplasm. This finding is consistent with the hypothesis that a significant fraction of the sodium in striated muscle fibers is bound to myosin. Since extracted myosin releases its associated alkali metal cations when it undergoes thermal denaturation (1), it was reasoned that myosin in the living cell might release its associated alkali metal cations during an irreversible shortening induced by a change in temperature.

To detect a release of cations from an internal source, the activities of sodium, potassium and hydrogen ions in the myoplasm were measured when the fibers shortened at 37-40^{o} C in a sodium free Ringer solution. The total concentrations of sodium and potassium in both control and experimental fibers were also measured. The main observation was that during the irreversible shortening of the fiber the activity of sodium in the myoplasm increased even though the total concentration of sodium in the experimental fibers decreased.

B. Methods

Experimental Procedure. In each experiment, four muscle fibers from a depressor muscle of Balanus nubilus were dissected free from one another. Before the start of an experiment, two fibers were cut away from the baseplate, washed for 10 seconds in isosmotic sucrose, blotted, and

placed in pre-weighed stoppered bottles for flame photometric analysis. One of the two remaining fibers was cannulated as described in the previous chapter, then transferred with its baseplate and companion fiber to the experimental chamber. The cation sensitive microelectrode was inserted through the cannulated tendon into the myoplasm until its sensitive tip was about 1 cm from the puncture zone (Figs. 2, 3). Deeper penetration was avoided because it increased the rate of breakage of the microelectrodes during contraction. The membrane potential of the fiber was always measured adjacent to the tip of the cation-sensitive microelectrode. During an experiment the open tip microelectrode was inserted and removed frequently but the cation-sensitive microelectrode was maintained in its initial position. The frequent puncturing of the membrane at the 1 cm level produced no significant decrease in the membrane potential. From the 1 cm level to the tendon level the membrane potential decreased less than 5 mV, indicating that the membrane at the fiber-tendon junction was well sealed around the cation-sensitive microelectrode.

In the experiments, the muscle fiber was exposed to solutions at different temperatures. The bath temperature was first lowered from 25 to 5° C, then was raised to 40° C. Between 5 and 35° C, the bath temperature was raised in increments of approximately 5° C by replacing the bathing solution with one at a higher temperature. Above 35° C, the bath temperature was increased at the rate of 0.5° C per minute by means of a glass-insulated heating coil. The bathing solutions were normal barnacle Ringer solution below 35° C, and sodium free isosmotic sucrose Ringer solution above 35° C (see Table 1 for the composition of these solutions). At 40° C, the bath temperature was held constant (39-42° C) until the experiment was terminated. The experiments involving measurements of potassium and hydrogen

activity were terminated after 15 minutes at 40° C, but the experiments involving measurements of sodium activity were continued until a_{Na} passed a maximum.

Membrane potential, cation activity, and fiber length were recorded at each new temperature. The bath temperature was measured by means of an iron-constantan thermocouple mounted in the chamber, and fiber length was measured by a millimeter scale. Since it was calculated that thermal equilibrium should be virtually complete 2 minutes after a solution change, all measurements were taken after this time interval. Between 35 and 40° C, the microelectrode potential and bath temperature were recorded continuously. During this period, three to five measurements of the membrane potential and fiber length were made.

The total concentrations of sodium and potassium in the experimental, companion and control fibers were determined by flame photometry in the experiments in which a_{Na} and a_K were measured. The fibers were dried, digested in concentrated nitric acid, neutralized with ammonia to prevent the formation of a flocculant precipitate, and then diluted to 10 ml before being analyzed on a Unicam SP900 spectrophotometer.

Microelectrodes. Details of the construction (2, 3) and calibration (Chapter IV) of the cation-sensitive microelectrodes have been described. For these experiments the sensitive tips of the microelectrodes were made relatively large (30μ x 200μ) to minimize breakage during contraction.

Eqns. [16] and [17] describe the behavior of the sodium and potassium microelectrodes. The equation which describes the behavior of the

pH sensitive microelectrode is

$$E_H = E_H' + S_H \log_{10} a_H \quad \text{.....} \quad [26]$$

where E_H is the measured potential (millivolts) of the microelectrode in a solution containing hydrogen ions at an activity a_H; E_H' and S_H are constants obtained by calibration. The potentials of the sodium and potassium sensitive microelectrodes were not altered when the pH was changed from 7 to 8. The potentials of the pH microelectrodes in standard pH solutions were not altered by gross changes in the Na^+ and K^+ content.

Since a_{Na} and a_K were not measured on the same muscle fiber, the following method of analysis was used to calculate a_{Na} from Eqn. [16]. The experiments presented in the previous chapter indicated that $a_K = 1.15\ C_K$ when the fiber is equilibrated at 25° C in the barnacle Ringer solution. Thus, a_K was estimated from the C_K values of the two control fibers. It was assumed that a_K remained constant throughout the experiment, an assumption justified by the results obtained from the potassium sensitive microelectrode (Fig. 3). Once a_K was estimated, a_{Na} could be calculated from Eqn. [16].

The values of a_K presented in Fig. 8 and Table IV were calculated from Eqn. [17]. The mean calculated results for a_{Na} were used to compensate for the imperfect selectivity of the potassium electrode. The errors introduced by this analytic technique should be small because a_{Na} is small relative to a_K, even though k_K (Eqn. [17]) is relatively large (0.5).

Each cation sensitive microelectrode was calibrated in the standard solutions before and after an experiment. If the calibrations varied by more than ±1 mV the experiment was rejected. The microelectrodes were

calibrated in the standard solutions at temperatures between 5 and 40° C, and the appropriate temperature corrections were applied to the experimental microelectrode potential readings. The precautions taken in the selection of open tip microelectrodes were identical to those described in Chapter IV, as was the recording apparatus.

The results were analyzed using Eqns. [24] and [25], which describe the separation of the sodium and water content of a single muscle fiber into a "free" and a "bound" fraction. To calculate the fraction of "bound" sodium, $B_{Na}/(C_{Na}V)$, from Eqn. [24], a numerical value must be assigned to α because both a_{Na} and a_K were not measured on the same fiber. The fraction of water free in the myoplasm was assumed to be the value obtained in the previous chapter ($\alpha = 0.57$). Results from pure solutions indicate that the activity coefficient should be relatively independent of temperature (4), but it must be assumed that α is independent of temperature. This latter assumption will be justified when the potassium results are discussed.

C. Results

Membrane Potential and Shortening. The mean results from the 27 experimental fibers are plotted in Fig. 6. When the temperature was raised from 7 to 25° C, the membrane potential increased by about 13 mV. This change was found to be reversible. As the temperature was raised above 30° C, the membrane potential decreased. Replacement of the barnacle Ringer solution by sodium free sucrose Ringer at 35° C ("S" in Fig. 6) did not produce any observable discontinuity in the downward trend of the membrane potential.

The length of the fibers remained constant up to about 37° C. Between 37 and 40° C, a strong spontaneous shortening occurred in all 27 fibers. This event did not seem to be causally related to the decrease in membrane potential for the following reasons. First, the membrane potential varied from 68 to 33 mV at the onset of the shortening. Second, relaxed, depolarized fibers in a 250 mM potassium, calcium free Ringer solution did not shorten until the temperature of this solution was raised to 37° C. The

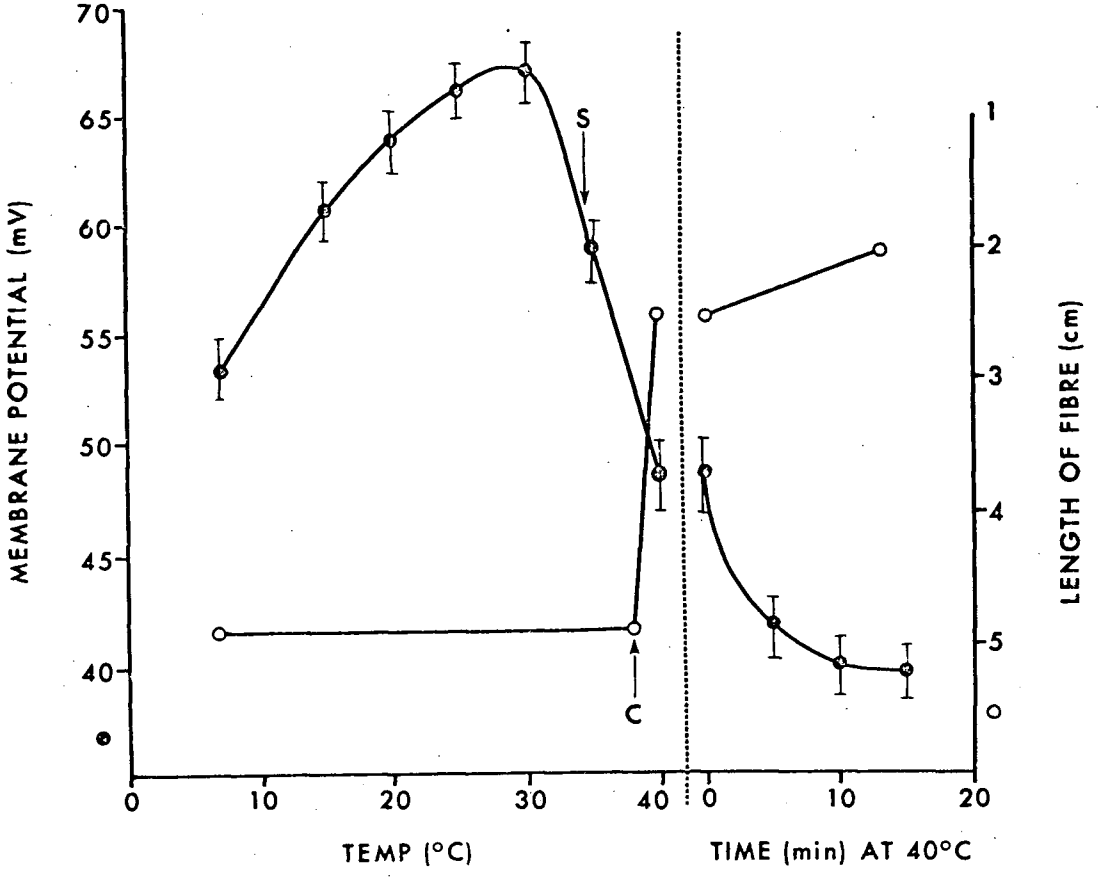

Fig. 6. Variation in the average membrane potential (closed circles) and fiber length (open circles) with temperature and with time at 40° C. At S the barnacle Ringer solution was replaced by sodium free sucrose solution. At C a spontaneous shortening occurred in all 27 fibers. Vertical bars through points are twice the standard error in length.

experimental fiber which supported the weight of the baseplate shortened about 50% in 1 minute, then continued to shorten another 10-20% over the next 15 minutes (Fig. 6). The companion fiber supported no weight and shortened from about 4.0 to 0.5 cm.

Changes in Intracellular Sodium. The results from a typical experimental fiber are shown in Fig. 7. In this fiber, the activity of sodium in the myoplasm, a_{Na}, increased from 0.003 to 0.006 as the temperature was raised from 7 to 35° C. The average results for this temperature interval,

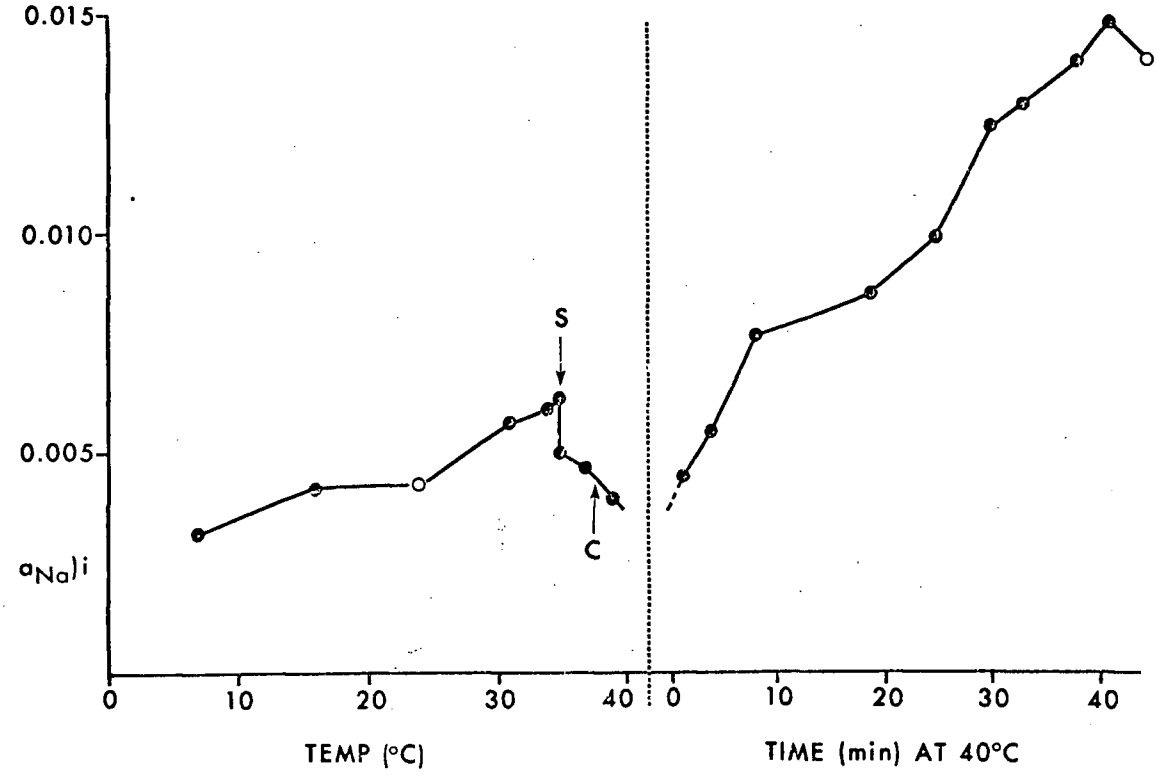

Fig. 7. Variation in the internal activity of sodium, $(a_{Na})_i$, of a typical muscle fiber as the temperature was increased to 40° C. The symbols S and C are defined in Fig. 6. Open circles represent the measurements in Table III.

however, revealed no significant increase in a_{Na}. When the normal Ringer solution was replaced by sodium free sucrose Ringer at 35° C ("S" in the figure), a_{Na} always decreased, indicating that sodium ions were moving out of the fiber. After the fiber shortened at 37-40° C, a_{Na} always increased. This increase was usually recorded within 1 minute and always within 5 minutes after shortening. In this experiment, a_{Na} increased from 0.004 to 0.015 in 44 minutes. In other experiments, maximum a_{Na} values were obtained earlier (Table III).

TABLE III

Sodium concentration* and activity * in single muscle fibers before (25° C) and after (40° C) shortening.

$(C_{Na})_{25}$	$(C_{Na})_{40}$	Minutes at 40° C †	$(a_{Na})_{25}$	$(a_{Na})_{40}$	$1 - (\alpha a_{Na}/\gamma C_{Na})$ ‡	
					25° C	40° C
0.058	0.046	11	0.009	0.014	0.86	0.73
0.076	0.053	21	0.007	0.018	0.92	0.70
0.060	0.060	45	0.009	0.037	0.87	0.46
0.085	0.052	38	0.010	0.017	0.90	0.71
0.123	0.051	14	0.005	0.007	0.96	0.88
0.099	0.069	40	0.005	0.012	0.96	0.85
0.163	0.099	18	0.011	0.023	0.94	0.79
0.080	0.123	27	0.008	0.010	0.91	0.93
0.061	0.055	44	0.004	0.015	0.94	0.76
0.081	0.085	35	0.006	0.011	0.93	0.89
0.089	0.069		0.007	0.016	0.92	0.77
±0.010§	±0.008		±0.001	±0.003	±0.01	±0.04

*Moles/kg fiber water.
†Time at 40° C for $(a_{Na})_{40}$ to reach a maximum value.
‡Fraction of bound Na calculated from Eqn. [24].
§Mean ± standard error of the mean.

Individual data for the 10 experimental fibers are presented in Table III. The average total sodium concentration of the two control fibers

analyzed at the start of the experiment is denoted as $(C_{Na})_{25}$. The sodium activity in the experimental fibers measured at 25° C (open circle, Fig. 7) is denoted as $(a_{Na})_{25}$. The $(C_{Na})_{40}$ and $(a_{Na})_{40}$ columns list the sodium concentrations and activities in the experimental fibers at the end of the experiment (open circle, Fig. 7). The last two columns of the table list the bound fractions of sodium calculated from the data by Eqn. [24].

A comparison of $(C_{Na})_{25}$ and $(C_{Na})_{40}$ indicates that the fibers lost sodium during the experiment. All the sodium loss probably occurred after the fibers were immersed in the sodium free sucrose Ringer solution. A comparison of $(a_{Na})_{25}$ and $(a_{Na})_{40}$ demonstrates the consistent increase in a_{Na} after the onset of shortening (Fig. 7). Since sodium could not enter the fiber from a sodium free bathing solution, and since it has been shown that sodium ions were in fact leaving the fiber, it may be concluded that either sodium ions were released from an internal site, or that there was a large reduction in the myoplasmic free water. Since this latter alternative demands a proportional increase in the activities of all myoplasmic cations, it can be ruled out because a_K remained relatively constant (Fig. 8). Thus, it may be concluded that the increase in a_{Na} results from a release of sodium from an internal site. The calculations in Table I show that the average percentage reduction in the fraction of "bound" sodium was 16 ± 4 (0.92 to 0.77).

Changes in Intracellular Potassium. The average results from seven experimental fibers are plotted in Fig. 8. When the fibers were heated from 7 to 35° C, there was no significant change in a_K. Immediately after shortening, a_K changed only from an average of 0.130 ± 0.006 to an average of 0.140 ± 0.008. For 15 minutes after shortening, a_K decreased slowly.

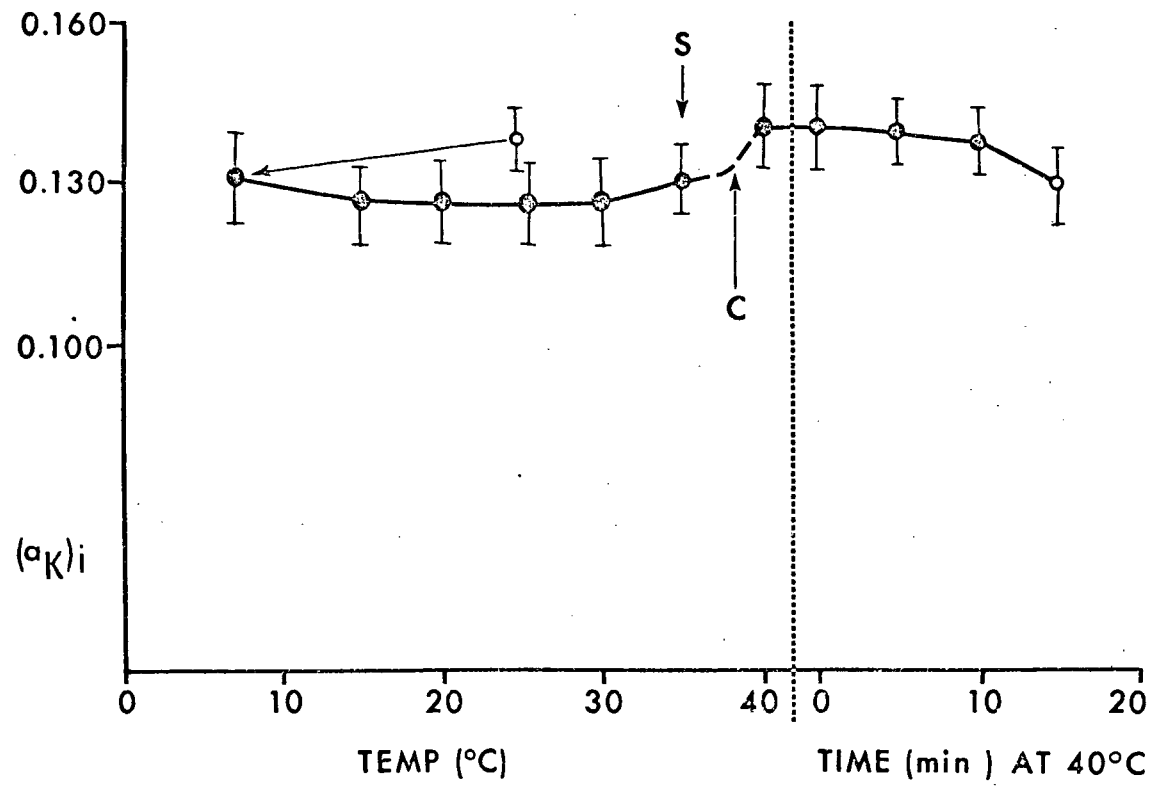

Fig. 8. Variation in the average internal activity of potassium, $(a_K)_i$, of seven fibers as the temperature was increased to 40° C. The shape of the curve (broken line) between 35 and 40° C was deduced from indvidual experiments. The symbols S and C are defined in Fig. 6. Open circles represent the measurements in Table IV. Vertical bars through points are twice the standard error in length.

Individual data for the seven fibers are given in Table IV. The columns denoted as $(C_K)_{25}$, $(C_K)_{40}$, $(a_K)_{25}$ and $(a_K)_{40}$ have the same significance as comparable columns for sodium in Table III. Comparison of the $(C_K)_{25}$ and $(C_K)_{40}$ values demonstrates that the fibers lost significant quantities of potassium ions, probably during the period of depolarization at 40° C (Fig. 6); yet comparison of the $(a_K)_{25}$ and $(a_K)_{40}$ values shows that

TABLE IV

Potassium concentration* and activity* in single muscle fibers before (25° C) and after (40° C) shortening.

$(C_K)_{25}$	$(C_K)_{40}$	$(a_K)_{25}$	$(a_K)_{40}$†
0.176	0.144	0.123	0.146
0.184	0.155	0.154	0.133
0.174	0.167	0.150	0.127
0.160	0.134	0.108	0.105
0.162	0.124	0.137	0.121
0.172	0.141	0.150	0.133
0.163	0.147	0.147	0.150
0.170 ±0.003‡	0.145 ±0.005	0.138 ±0.006	0.131 ±0.006

*Moles/kg fiber water.
†After 15 minutes at 40° C.
‡Mean ± standard error of the mean.

the activity of potassium in the myoplasm was not significantly altered.

There are two possible explanations for these results; either potassium ions are released into the myoplasm from an internal site, or water is removed from the myoplasm to an internal site. If it is assumed that the first explanation is correct, and further assumed that all the bound potassium ions are released during shortening, Eqn. [19] predicts that only 10% of the total fiber potassium need be bound and released to explain the results. Alternatively, if it is assumed that the second explanation is correct, and further assumed that there is no binding of potassium either at the beginning or the end of the experiment, Eqn. [19] predicts that the fraction of bound water must increase by 10% at the expense of the myoplasmic free water. It should be emphasized that neither of these possibilities alters the conclusions from the experiments in which a_{Na} was measured.

Changes in Intracellular Hydrogen. The average results from ten

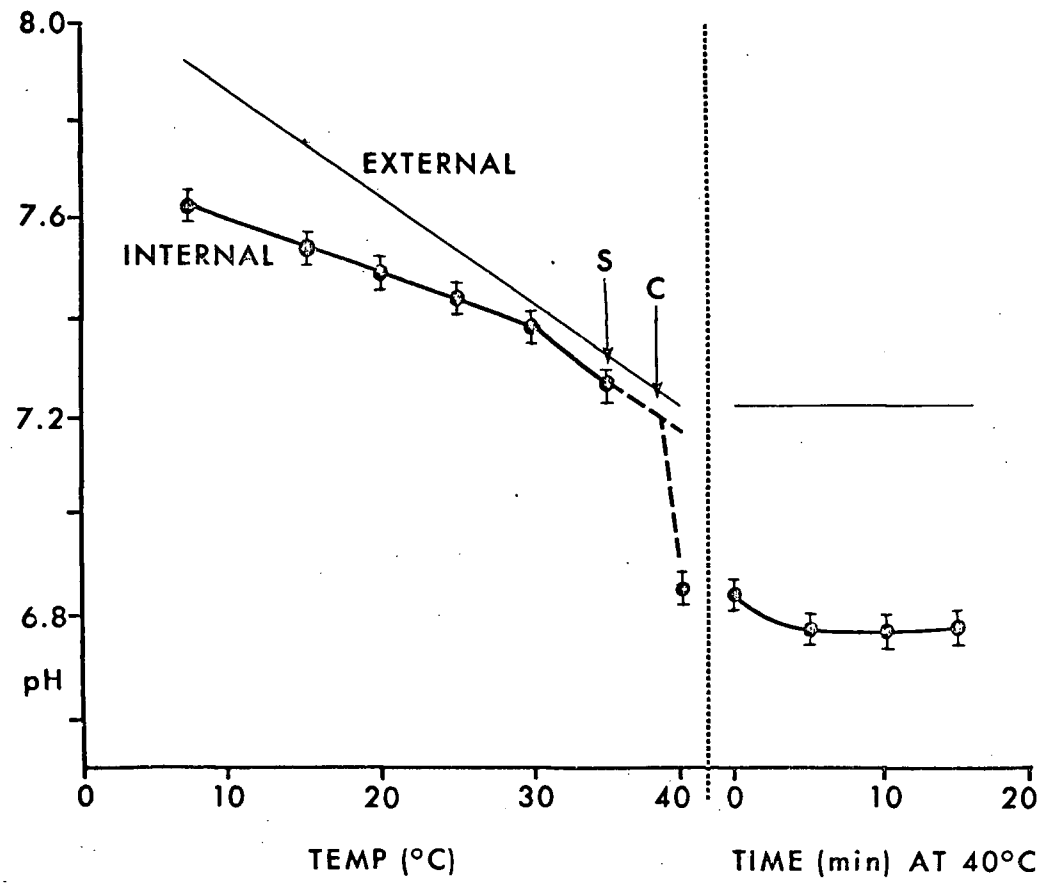

Fig. 9. Variation in the average pH of the myoplasm (internal) of 10 fibers as the temperature was increased to 40° C. The variation of pH in the bath solution with temperature (external) was also measured and is shown as a thin line. The shape of the curve (broken line) between the experimental points at 35 and 40° C was deduced from individual experiments. The symbols S and C are defined in Fig. 6. Vertical bars through points are twice the standard error in length.

experimental fibers in which the pH was measured are plotted in Fig. 9. Between 7 and 30° C a linear decrease in pH was observed in both the myoplasm and the bath solution. In both cases, the pH changes were probably due to the temperature dependence of the buffer systems. It is unlikely that the myoplasmic pH changes were due to an inward diffusion of hydrogen ions because of the short time interval between measurements. Above 30° C the

rate of change of the internal pH increased slightly. At 35° C, no change in internal pH was observed when the bath solution was changed to sodium free sucrose Ringer. When shortening occurred ("C" in the figures) the pH dropped suddenly in all ten fibers. The average drop in pH was from an extrapolated value of 7.17 at 40° C to a measured value of 6.85 ± 0.04 at 40° C. Ten minutes after the onset of shortening the pH reached an average mininum value of 6.77 ± 0.05. These results leave little doubt that a sudden reduction in myoplasmic pH occurred at the onset of shortening.

D. Discussion

Variations in Membrane Potential. The changes in membrane potential with temperature (Fig. 6) warrant comment even though they are secondary to the main findings. From 7 to 25° C, the average membrane potential increased by 13.0 mV, yet there was no significant change in either a_{Na} or a_K. Assuming that membrane permeability to sodium and potassium remains constant, it may be calculated from the Goldman equation (5) that the membrane potential should only increase 3.4 mV over this temperature range. This anomalous dependence of membrane potential on temperature, also observed by Frumento (6) in frog muscle, may be due to either a decrease in the cation permeability ratio (P_{Na}/P_K) or the existence of an electrogenic pump (7-11).

Intracellular Sodium and Water "Binding". The activity and concentration values measured here are similar to those reported in the previous chapter, hence they confirm qualitatively the original conclusions about sodium and water "binding". The calculated mean fraction of "bound" sodium was 0.92 ± 0.01 in the present experiments and 0.84 ± 0.00 in the previous experiments (Chapter IV). The latter estimate is probably more reliable because it was calculated from a_{Na}, a_K, C_{Na} and C_K measurements on

the same muscle fiber.

The calculated mean fraction of "bound" water, $(1-\alpha)$, was 0.27 ± 0.03 in the present experiments and 0.41 ± 0.01 in the previous experiments (Chapter IV). Both calculations were based on the assumptions leading to Eqns. [24] and [25]. The previous estimate is believed to be more reliable for reasons similar to those given for the bound sodium fraction. It is possible, however, that the discrepancy between the results is real because the barnacles were collected from different locations in different seasons and stored in sea water at different temperatures (4 and 10° C). Hinke (3), in a recent independent series of measurements also found that the fraction of "bound" water in barnacles taken from the second location and stored at 10° C was low; $(1-\alpha) = 0.26$. (Note that if α is assumed to have a value of 0.73 instead of 0.59, the fraction of "bound" sodium calculated from the data of Table III changes by only a small amount; from 0.92 to 0.90.)

Another possibility is that an electrical error was introduced in the cation sensitive or open tip microelectrode measurements either in the present or the previous experiments. It can be shown, for example, that a 6 mV error in the potassium potential (E_K in Eqn. [17]) could produce the discrepancy in the calculated "bound" water fractions whereas a 6 mV error in the sodium potential (E_{Na} in Eqn. [16]) would not alter the magnitude of the "bound" sodium fraction. Such an error is unlikely, however, because of the careful selection of open-tip microelectrodes, and the rejection of experiments when the cation-sensitive microelectrode potentials in standard solutions deviated by more than 1 mV.

Release of Bound Sodium. It was demonstrated that an increase in the mean a_{Na} occurred when the muscle fibers shortened due to exposure to

temperatures between 37 and 40° C (Fig. 6 and Table III). This increase was not due to an inward diffusion of sodium ions because no sodium ions were in the bathing solution. It is unlikely that the increase resulted from an electrical artifact because during shortening the potential of the potassium sensitive microelectrode did not change significantly (Fig. 8) and the potential of the hydrogen sensitive microelectrode changed more rapidly than that of the sodium sensitive microelectrode (Figs. 9 and 7). As a_{Na} decreases in sucrose Ringer at 25° C (Fig. 18), it may be concluded that the increase in a_{Na}, and the corresponding 16% reduction in $B_{Na}/(C_{Na}V)$, observed in the present experiments are causally related to the shortening induced by a change in temperature.

It may also be concluded that the increase in a_{Na}, or the release of "bound" sodium, is characteristic of a temperature induced shortening, but probably not of a normal contracture. When barnacle muscle fibers were contracted at 25° C by exposure to a solution containing 0.064 M KCl, a_{Na} was found to be 98 ± 4% (9 experiments) of the initial value (a_{Na} of the same fiber bathed in normal Ringer) after 4 minutes in the high potassium solution, and 97 ± 6% (9 experiments) of the initial value after 8 minutes in the high potassium solution (preliminary experiments).

Myoplasmic pH. Since the pH of the myoplasm was 7.43 and the pH of the bathing solution was 7.54 at 25° C, the equilibrium potential for the hydrogen ion was 6 mV. The average membrane potential at this temperature, however, was 66 mV. Thus, hydrogen ions are not distributed between the myoplasm and the bathing solution according to the Nernst equation. Similar results have been reported for muscles of the crab (12) and frog (13), although not for rat skeletal muscles (14).

A large, rapid decrease in myoplasmic pH occurred when the muscle

fibers shortened. The rapidity of the decrease indicates that it was not due to hydrogen ions diffusing into the fiber from the bathing solution. Even if hydrogen ions did diffuse into the fiber, no change in pH would be observed because of the large buffer capacity of the myoplasm. This was demonstrated in experiments performed in depolarized fibers (12, 13), and also by the pH experiments reported in Chapter VI of this thesis. Therefore, the pH change observed at 37-40° C in these experiments was due to a change in the pK of the organic buffers in the myoplasm. Since the contractile proteins constitute the main organic buffer, the change in the pK values probably signifies a disruption of the myofilaments.

Location of Bound Sodium. The basic hypothesis of this report is that a significant fraction of the sodium in striated muscle fibers is bound to myosin. It is known (1) that extracted myosin undergoes thernal denaturation at a lower temperature (37° C) than extracted actin (50° C). It is also known that when extracted myosin is exposed to temperatures above 37° C it releases associated alkali metal cations (1) and ATP molecules (15). Thus, the object of these experiments was to disrupt the thick or A filaments in an intact fiber and observe any changes in the fraction of "bound" sodium.

There are several reasons for believing that either the A or the I filaments were structurally altered at 37-40° C: (i) an irreversible shortening occurred at this temperature, (ii) calcium ions were not required in the bathing solution for this shortening, (page 67), (iii) the shortening occurred independently of changes in the membrane potential and (iv) a large, rapid decrease in pH accompanied the shortening.

Experiments performed on glycerinated muscles, as well as on ex-

tracted proteins, indicate that it was the A and not the I filaments that were disrupted in these experiments at 37-40° C. Thermal disruption of the A filaments was observed by Aronson in glycerinated muscles (16). After heating the muscles for 2 minutes at a critical temperature (which varied from 43.5° C for frog to 51° C for mouse muscle) he observed a decrease in the birefringence of the muscle, and a loss in the A filament structure as seen under the electron microscope. The shortening and the decrease in the myoplasmic pH of barnacle muscle fibers exposed to temperatures of 37-40° C are probably related to the thermal disruption of the A filaments that Aronson observed. The myosin molecules, themselves, however, need not be completely denatured when the breakdown of the A filaments occurs. In fact, available evidence indicates that a slow denaturation of the myosin molecules should occur at 37° C, for it takes about 1 hour for the viscosity of extracted myosin to double at this temperature (1). Consistent with this fact is the observation that in barnacle muscles the release of "bound" sodium at 37-40° C occurs over a period of about 1/2 hour.

The results presented in this chapter are thus in excellent agreement with the hypothesis that much of the sodium in an intact striated muscle fiber is bound to myosin. It must be admitted, however, that the data could be interpreted in a different manner. It could be argued that the increase in a_{Na} observed at 37-40° C was due to a release of sodium from intracellular compartments such as nuclei, mitochondria or the cisternae of the sarcoplasmic reticulum. This explanation seems unlikely for the following reasons. First, a_{Na} does not increase following a potassium induced contracture at 25° C (preliminary experiments). Thus, it must be argued that the membranes are disrupted by the temperature, not the shortening, in such a way as to allow sodium to exit from the compartments. Note

however, that a_{Na} always decreased between 35° C and the temperature at which shortening occurred (Fig. 7). The membrane potential varied between 68 and 33 mV at the time of shortening, but no increase in a_{Na} was noted until after the shortening occurred. Thus, there is a far better correlation between the irreversible shortening and the increase in a_{Na} than there is between the membrane potential and the increase in a_{Na}.

In summary, it has been demonstrated that a significant release of "bound" sodium occurs following an irreversible shortening induced by a temperature change. It was argued that this shortening was related to a disruption of the thick or A filaments, an indication that at least part of the fraction of "bound" sodium in striated muscle fibers is associated with myosin.

CHAPTER VI

OPTICAL DENSITY CHANGES OF FIBERS IN SODIUM FREE SOLUTIONS

A. Introduction

The optical experiments reported below were undertaken to obtain further evidence that some of the sodium in striated muscle fibers which is unavailable to a sodium sensitive microelectrode is bound rather than compartmentalized. The idea of using light scattering measurements to detect the binding of ions to macromolecules is not new. Edsall and his coworkers (1) utilized this technique to measure the binding of chloride, calcium and thiocyanate ions to protein serum albumin.

Light is scattered from a colloidal solution because of local fluctuations in refractive index. These are due to fluctuations in concentration caused by random thermal motion, which in turn are counteracted by the increase in free energy which arises from the fluctuation. Fluctuations in concentration contribute proportionally to the square of the resulting fluctuations in refractive index. Edsall et al (1) extended the fluctuation theory to multicomponent systems containing charged macromolecules, while Doty and Steiner (2) approached the problem via the interference theory. Both these approaches are discussed in a review article (3).

The theoretical basis for investigating the binding of ions to macromolecules by light scattering measurements is illustrated by Eqn. [27]

$$Hc/\tau = 1/M + (Z^2c)/(2m_3M^2) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots [27]$$

which describes the relation between the turbidity, τ, of an ideal three component system (consisting of water, salt and macro-ion salt) and the net

charge, Z, on the macro-ion. The term H represents a collection of optical constants and varies inversely with the fourth power of the wavelength. The terms c and M represent respectively the concentration and molecular weight of the macro-ion while m_3 represents the concentration of the micro-ion of opposite charge. This simple relation can be derived by applying the condition of Donnan equilibrium to a system for which the ionic strength is not too low and by considering only electrostatic interactions (1, 2, 3). It is apparent from Eqn. [27] that increasing the net charge, Z, on the macro-ion decreases the turbidity, τ, of the solution. It was reasoned that if sodium was bound to negatively charged macromolecules within the barnacle muscle, bathing the fiber in a sodium free solution would cause sodium to move off the binding sites and out of the fiber. If no ion replaced sodium on the binding sites, the net charge of the macromolecules would increase, and the turbidity of the fiber would decrease. Thus, muscle fibers bathed in sodium free solutions were examined for any decrease in turbidity.

B. Methods

Determination of Activities and Concentrations. The sodium sensitive microelectrodes were calibrated in solutions containing both sodium and potassium (Chapter IV) as well as in solutions containing 0.200 M KCl, 0.010 M NaCl and either 0.004 or 0.040 M LiCl. The hydrogen sensitive microelectrodes were calibrated in standard buffers of pH 7 and 8. The microelectrodes were calibrated before and after each experiment and the results were rejected unless the calibrations coincided (±1 mV). Conventional open tip microelectrodes filled with 3 M KCl were used to measure the membrane potential of the fibers. (The membrane potential was of course subtracted from the potential recorded from the sodium sensitive microelectrode in the

TABLE V

Solutions (M)

	Normal* Ringer	Sucrose Ringer	Tris Ringer	Calcium free Ringer	Potassium Ringer	Lithium Ringer
NaCl	.450	.000	.000	.480	.000	.000
$CaCl_2$	.020	.020	.020	.000	.000	.020
$MgCl_2$	.010	.010	.010	.010	.010	.010
KCl	.008	.008	.008	.008	.488	.008
Tris Cl	.025	.025	.475	.025	.025	.025
LiCl	.000	.000	.000	.000	.000	.450
Sucrose	.000	.650	.000	.000	.000	.000

*The pH of every solution in this table was 7.6. Normal Ringer buffered to pH = 9.6 with tris or to pH = 5.5 with CO_2 was also used for some experiments.

myoplasm.) The precautions taken in the selection of these microelectrodes, the calibration procedure and the recording apparatus were identical to those described in Chapter IV.

The sodium sensitive microelectrodes were used to measure the activity of sodium in the myoplasm of fibers bathed in sucrose and lithium Ringer. The hydrogen sensitive microelectrodes were used to measure the pH of the myoplasm of fibers bathed in pH = 9.6 and pH = 5.5 Ringer. The compositions of the bathing solutions used in these experiments is given in Table IV.

Analytic measurements were not made on fibers used for microelectrode experiments. Separate experiments were conducted to determine the movement of alkali metal cations when the fibers were bathed in lithium and

sucrose Ringer. Seven fibers attached to a single baseplate were dissected free from one another in normal Ringer. Two fibers were taken as controls; they were blotted, swirled for 30 seconds in iso-osmotic sucrose, blotted again, then placed in pre-weighed bottles. The remaining 5 fibers were suspended by their tendons in the bath which contained either sucrose or lithium Ringer, removed at 1, 3, 5, 10 and 25 minutes and handled in the same manner as the controls. The fibers were then dried and digested in nitric acid. The resulting solution was neutralized with ammonia, diluted to 10 ml and analyzed for sodium and potassium (and lithium if applicable) on a Unicam SP 900 flame spectrophotometer.

Determination of Relative Optical Density. As shown in Fig. 10, a single muscle fiber was suspended by its tendon in a clear perspex chamber containing normal Ringer. The baseplate of the fiber was firmly embedded in plasticine and the fiber was stretched to about 120% of its resting length. The chamber was then positioned in a Beckman B spectrophotometer (Fig. 10) and the optical density ($O.D._o$) measured for 10 minutes to ensure its constancy. In the first series of experiments, the slit width remained constant while the O.D. was determined at various wavelengths. Normal Ringer was then replaced by sucrose Ringer and the O.D. measured for 25 minutes at a single wavelength. After this period of time the O.D. was again measured at various wavelengths. Sucrose Ringer was then replaced by normal Ringer, and a final scan of wavelengths made after 25 minutes. In all other experiments, O.D. measurements were made only at 850 mμ.

Some pertinent experimental details are as follows. The spectrophotometer was equipped with a constant voltage transformer, and was always turned on one hour prior to an experiment. The wavelength dial was

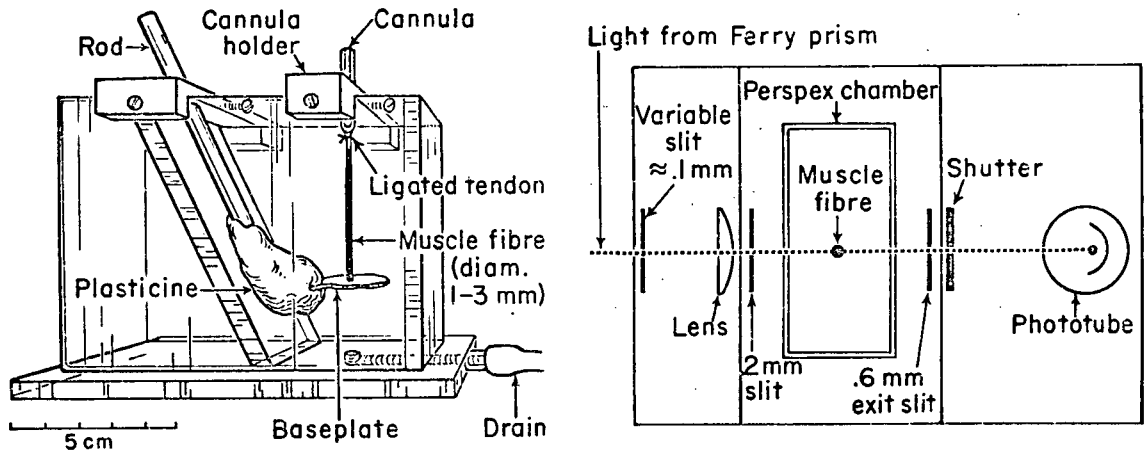

Fig. 10. Diagram of a single muscle fiber positioned in the perspex bathing chamber (left), and plan view of the optical pathway (right).

calibrated with a mercury lamp. All bathing solutions were filtered before use to remove dust particles. The chamber filled with the bathing solution was used as the blank at all wavelengths. If the final and initial blank readings did not coincide (±2%), the experimental results were rejected. The width of the beam was always less than 1/2 the width of the fiber, and usually much less because large, flat fibers were selected for these experiments. All experiments were conducted at room temperature (23-25° C).

Limitations of the Experimental Apparatus and Method. The optical

density, O.D., is defined in Eqn. [28]

$$O.D. = -\log_{10}(I/I_o) \quad \text{.....[28]}$$

where I/I_o represents the transmittance, or the ratio of the transmitted to the incident intensity. The turbidity, τ, is defined as the ratio of the total light scattered to the product of the incident intensity of light and volume of solution (or muscle) which scatters light. It may be expressed as an extinction coefficient, and when absorption is negligible,

$$\tau = -(2.303/\ell) \log_{10}(I/I_o) \quad \text{.....[29]}$$

where ℓ is the optical path length of the beam in the muscle fiber. It is apparent from Eqns. [28] and [29] that in the absence of absorption the turbidity of the fiber relative to its initial value in normal Ringer is equal to the O.D. of the fiber relative to its initial value in normal Ringer. In the presence of generalized absorption, Eqn. [29] must be modified, and to a first approximation

$$\beta + \tau = -(2.303/\ell) \log_{10}(I/I_o) \quad \text{.....[30]}$$

where β is an extinction coefficient due to absorption. A simple algebraic manipulation can then be made to show that the change in relative turbidity is always greater than the change in the relative O.D. (Appendix 1). Thus, a possible constant absorption was accepted as an error because the change in the turbidity of the fiber was underestimated. This error will decrease as the wavelength of incident light increases because generalized absorption decreases with increasing wavelength.

In these experiments, the height of the light beam was 19 mm at the last entrance slit, about 12 mm at the muscle and 6 mm at the exit slit. The theory of light scattering assumes parallel indicent light, but many

investigators use converging light for scattering measurements, and as Stacey (4) has stated "in practice the error due to the use of converging light has not been a serious one." A more serious error arises from the fact that the light beam could not be precisely focussed. The width of the light beam increased from less than .2 mm at the entrance slit to .6 mm at the exit slit. Thus, the diverging width of the beam forced the exit slit to have a width of .6 mm. A smaller exit slit would have collected less light scattered through very small angles. This error, however, implies that the measured change in O.D. is less than the change in τ, hence it was accepted (Appendix 2).

In any highly turbid medium like a muscle fiber, secondary scattering complicates the interpretation of light scattering measurements. When the scattering particles have one dimension greater than 1/20 of the wavelength of the incident light, internal interference also occurs. Doty and Steiner (5) have discussed this phenomenon in an article on the spectrophotometric measurement of turbidity. Although both secondary scattering and internal interference must be considered when the absolute turbidity of a solution is measured, these factors probably may be ignored when the relative turbidity of muscle fibers in various solutions is measured. The effect of internal interference on the turbidity should remain constant throughout the experiment. The existence of secondary scattering implies that the actual change in turbidity is greater than the change in O.D. that is measured and this effect can be minimized by making measurements at long wavelengths.

C. Results

Sucrose Ringer. Fig. 11 summarizes the flame photometric measure-

ments of the sodium and potassium concentrations of fibers soaked for varying times in sodium free sucrose Ringer. The activity of sodium in the myoplasm, a_{Na}, is also shown. Since the sodium sensitive microelectrodes were not absolutely selective for sodium a small correction for the activity of potassium in the myoplasm, a_K, was applied to the electrode readings (Eqn. [16]). The value of a_K was not measured in these experiments, but was

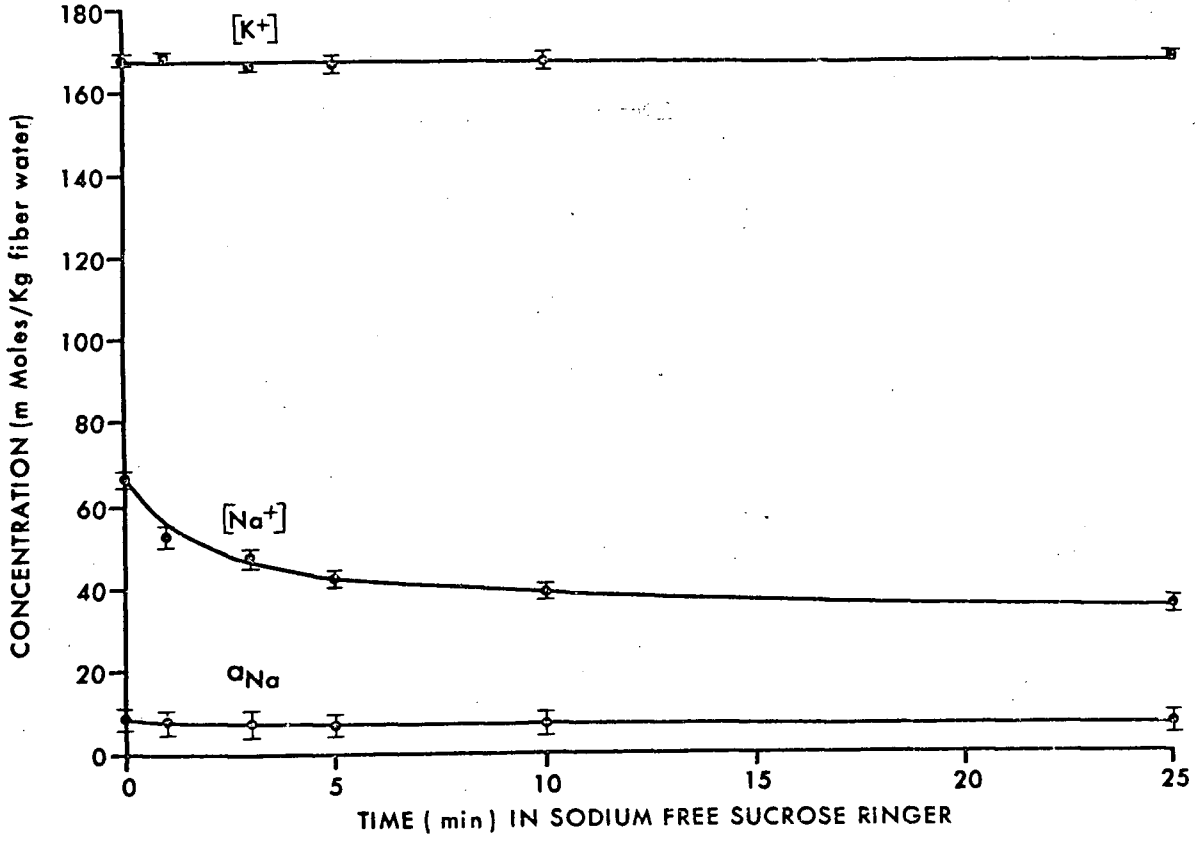

Fig. 11. The total concentrations of sodium and potassium in fibers bathed in sodium free sucrose Ringer. Initial points are the average of measurements from 20 fibers. Other points are the average of measurements from 10 fibers. The activity of sodium in the myoplasm, a_{Na}, as measured by a sodium sensitive microelectrode, is also shown. The number of experiments, n, was 5. The vertical bars are twice the S.E. in length.

estimated from the measured average concentration of potassium (Fig. 11) using the empirical relation $a_K = .87[K]$ (Chapter V).

From the data presented in Chapter IV, it was calculated that the percentages of total fiber sodium unavailable to a microelectrode when fibers were bathed in normal and sucrose Ringer were 84% and 81% respectively. In comparison, the calculated values from the data of Fig. 11 are 85% and 80%. These estimates do not take into account the large extracellular space which was recently discovered in single barnacle muscle fibers (6). This compartment was found to comprise about 5% of the total fiber volume by two independent techniques, and it presumably contains about .030 moles of sodium/kg fiber water (6). If the total concentration of sodium in fibers bathed in normal Ringer, .066 moles/kg fiber water (Fig. 11), is corrected for the sodium in the extracellular compartment, the percentage of intracellular sodium unavailable to the microelectrode in normal Ringer becomes 72%. This value increased to 80% when the fibers were bathed for 25 minutes in sucrose Ringer. Thus, the previous observation (Chapter IV) that the fraction of total fiber sodium unavailable to the microelectrode decreased slightly when the fibers were bathed in sucrose Ringer is explained by the large extracellular space of the fibers.

The transmittance of single muscle fibers is graphed in Fig. 12 as a function of wavelength. The lower curve is the transmittance of the fibers in normal Ringer and the upper curve is the transmittance of the same fibers after 25 minutes in sucrose Ringer. The apparent discontinuity in the curves between 600 and 650 mμ is an artifact which arises because a different group of fibers had to be used for the wavelengths below 600 and above 650 mμ. No discontinuity was observed in the transmittances of two

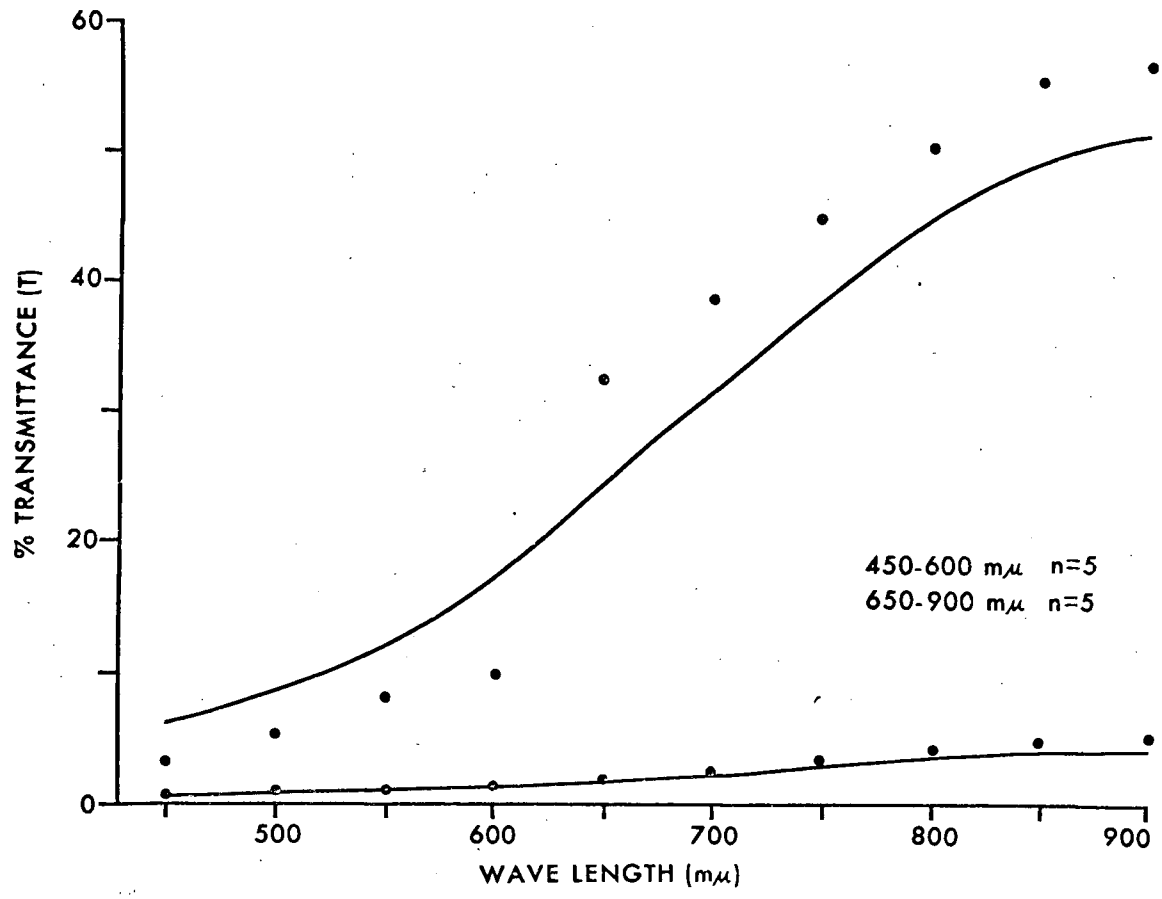

Fig. 12. The transmittance of single muscle fibers in normal Ringer (lower curve) and after 25 minutes in sucrose Ringer (upper curve) as a function of wavelength.

fibers which were scanned from 600 to 900 mμ in normal and sucrose Ringer. It is apparent from Fig. 12 that at a given wavelength the transmittance of a muscle fiber is greater in sucrose than in normal Ringer. Note also that in either sucrose or normal Ringer, the transmittance increases with wavelength.

In an ideal solution, the turbidity varies inversely with the fourth power of the wavelength. This implies that a plot of the log of the turbidity (or O.D.) against the log of the wavelength will yield a straight

line with a slope of -4. This relationship was not observed for single muscle fibers, although the data of Fig. 12 illustrates that the transmittance does increase with the wavelength. The deviation from the inverse fourth power relationship is presumably due to the existence of internal interference, secondary scattering and absorption. Internal interference alone can change the inverse fourth power relationship between turbidity and wavelength to an inverse square relationship (4).

The transmittance of a muscle fiber is dependent on the thickness

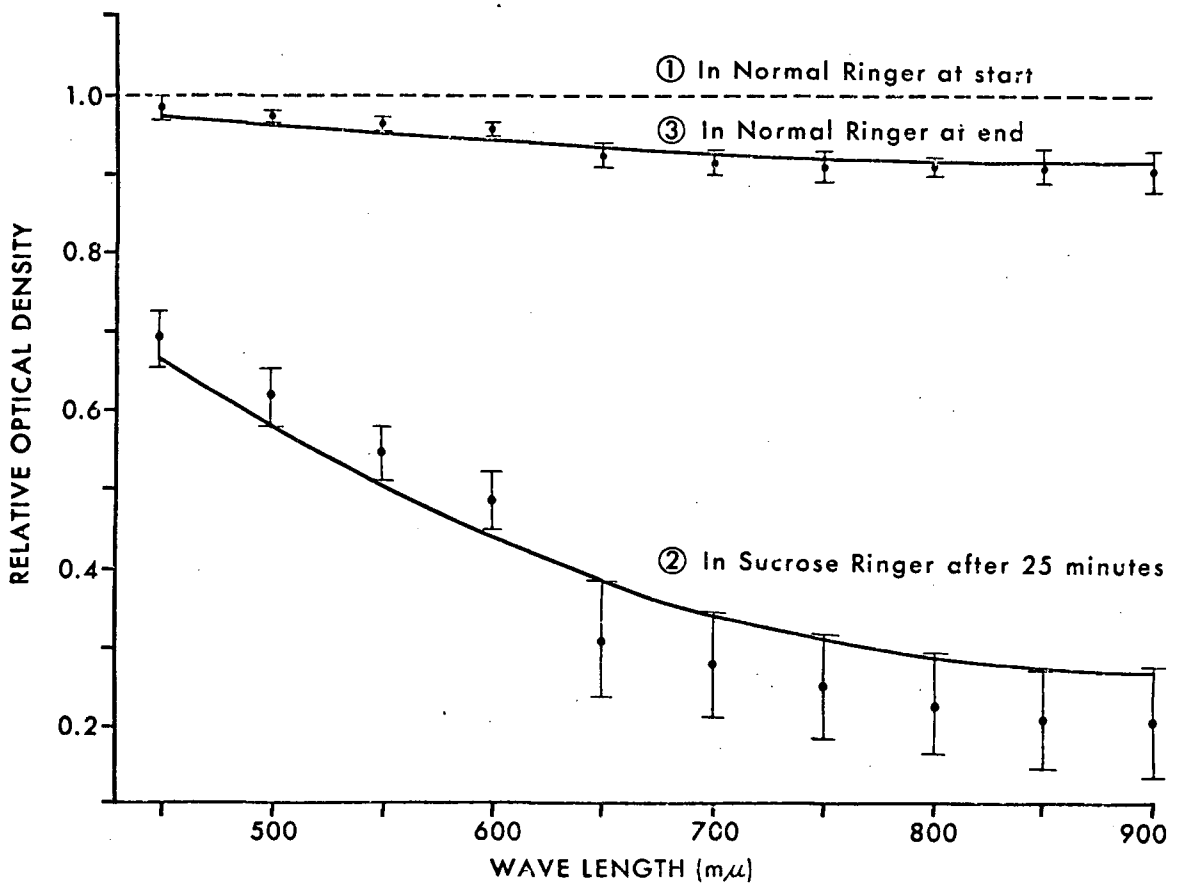

Fig. 13. The optical density, O.D., of single muscle fibers relative to the initial value of the O.D. in normal Ringer as a function of wavelength. The data in curve 2 are from fibers bathed for 25 minutes in sucrose Ringer; the data in curve 3 are from the same fibers 25 minutes after they were returned to normal Ringer.

of the fiber or optical path length. This dependence on optical path length can be avoided by considering the relative optical density (O.D.) of a fiber (Fig. 13) rather than the transmittance. The relative O.D. drops from an initial value of 1 (curve 1, Fig. 13) in normal Ringer to low values (curve 2, Fig. 13) after 25 minutes in sucrose Ringer. The largest change occurs at the longest wavelength where the errors due to possible absorption, scattering through small angles and secondary scattering are minimal. Curve 3, Fig. 13, shows that this phenomenon is almost completely reversible. When normal Ringer was returned to the chamber, the relative O.D. of the fibers increased within 25 minutes to over 90% of the initial value.

Tris Ringer. In Fig. 14 the relative O.D. (at 850 mμ) of single muscle fibers bathed in sodium free, tris substituted Ringer is graphed as a function of time. The time for the O.D. of the fibers to reach a constant value in either tris or sucrose Ringer was identical (10-15 min., Fig. 14) but the magnitude of the decrease was not as great in tris as in sucrose Ringer. When normal Ringer was returned to the chamber, the O.D. of the fibers increased rapidly to its initial value (Fig. 14). The recovery of the O.D. of fibers bathed in sucrose Ringer was also noted to be more rapid than the initial decrease in the O.D.

Potassium Ringer. In Fig. 15 the relative O.D. (at 850 mμ) of single muscle fibers bathed in sodium free, calcium free, potassium substituted Ringer is graphed as a function of time. It was necessary to presoak the fibers in a calcium free solution, and remove calcium from the sodium free, potassium substituted solution to prevent the contracture of the fibers. The O.D. of the fibers was first measured in normal Ringer, then in calcium free Ringer. Exposure to calcium free Ringer for 25 minutes

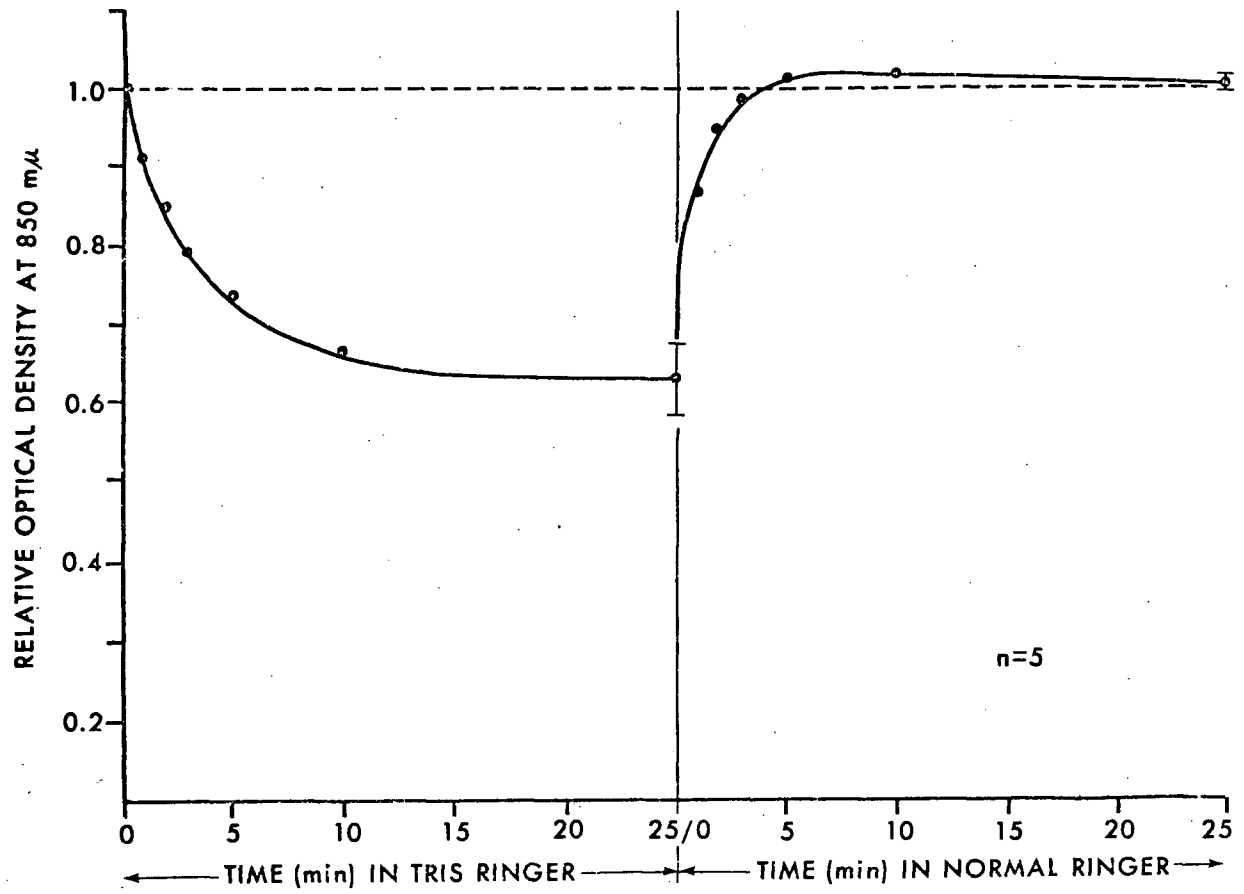

Fig. 14. The relative O.D. (850 mμ) of single muscle fibers bathed in tris Ringer and then in normal Ringer.

lowered the O.D. slightly (note the relative O.D. at zero time in Fig. 15). When this solution was replaced by potassium Ringer, the O.D. decreased as shown in Fig. 15. This decrease was greater than the decrease in tris Ringer, but less than the decrease in sucrose Ringer. The recovery of the O.D. was very slow compared to the recovery of the O.D. of fibers initially bathed in sucrose or tris Ringer. The O.D. was still increasing slightly after 50 minutes in normal Ringer (Fig. 15).

As potassium is more permeable than sodium, one might expect that

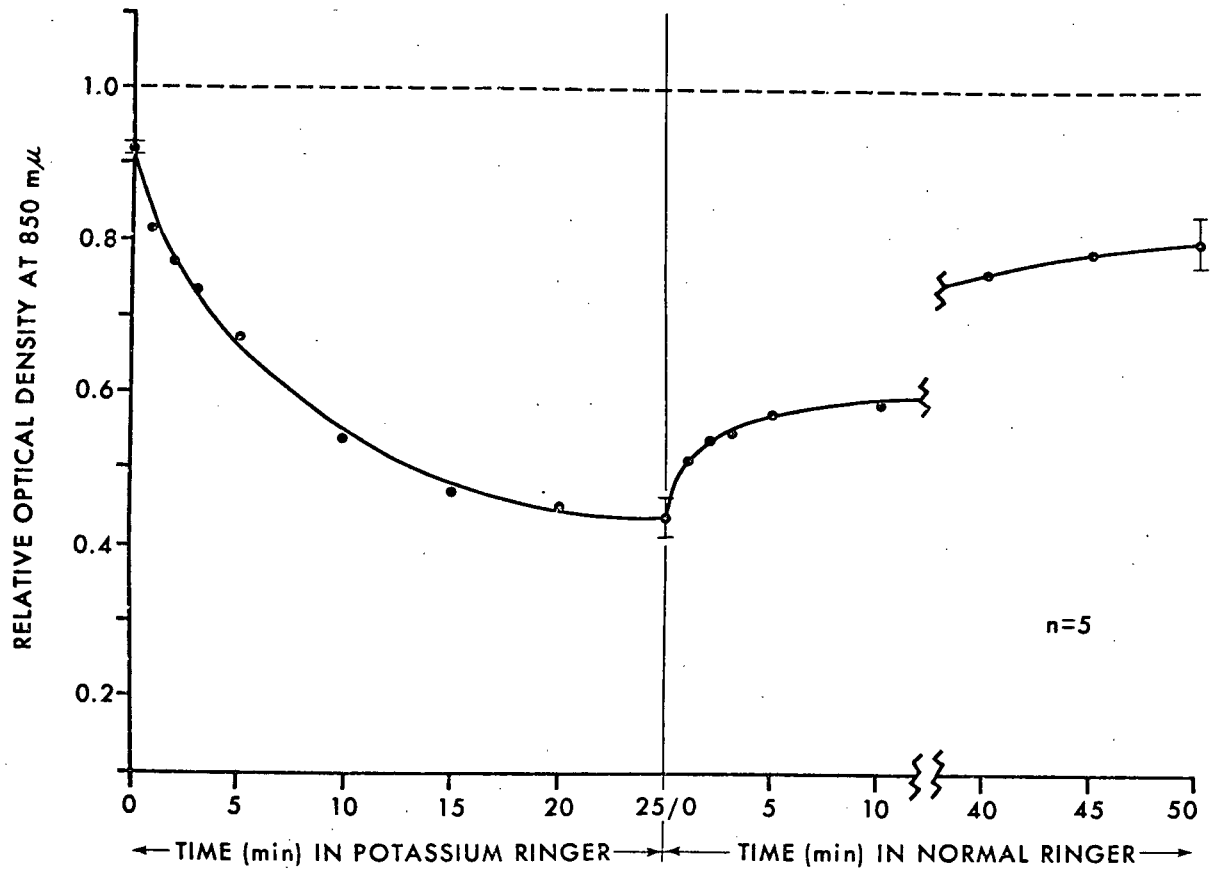

Fig. 15. The relative O.D. (850 mμ) of single muscle fibers bathed in potassium Ringer and then in normal Ringer. The fibers were initially bathed for 25 minutes in calcium free Ringer.

a substantial increase in the volume of the fibers occurred when they were bathed in the potassium Ringer solution. The volume of the fibers did increase in this solution, but the increase was so slight as to be negligible. A control experiment indicated that the percentage water content of fibers bathed in normal Ringer was 76.6 ± 0.1 (n=5) whereas the percentage water content of fibers from the same muscle bathed for 25 minutes in calcium free Ringer, then for 25 minutes in potassium Ringer was 77.7 ± 0.2 (n=9).

Lithium Ringer. In Fig. 16 (upper curve) the relative O.D.

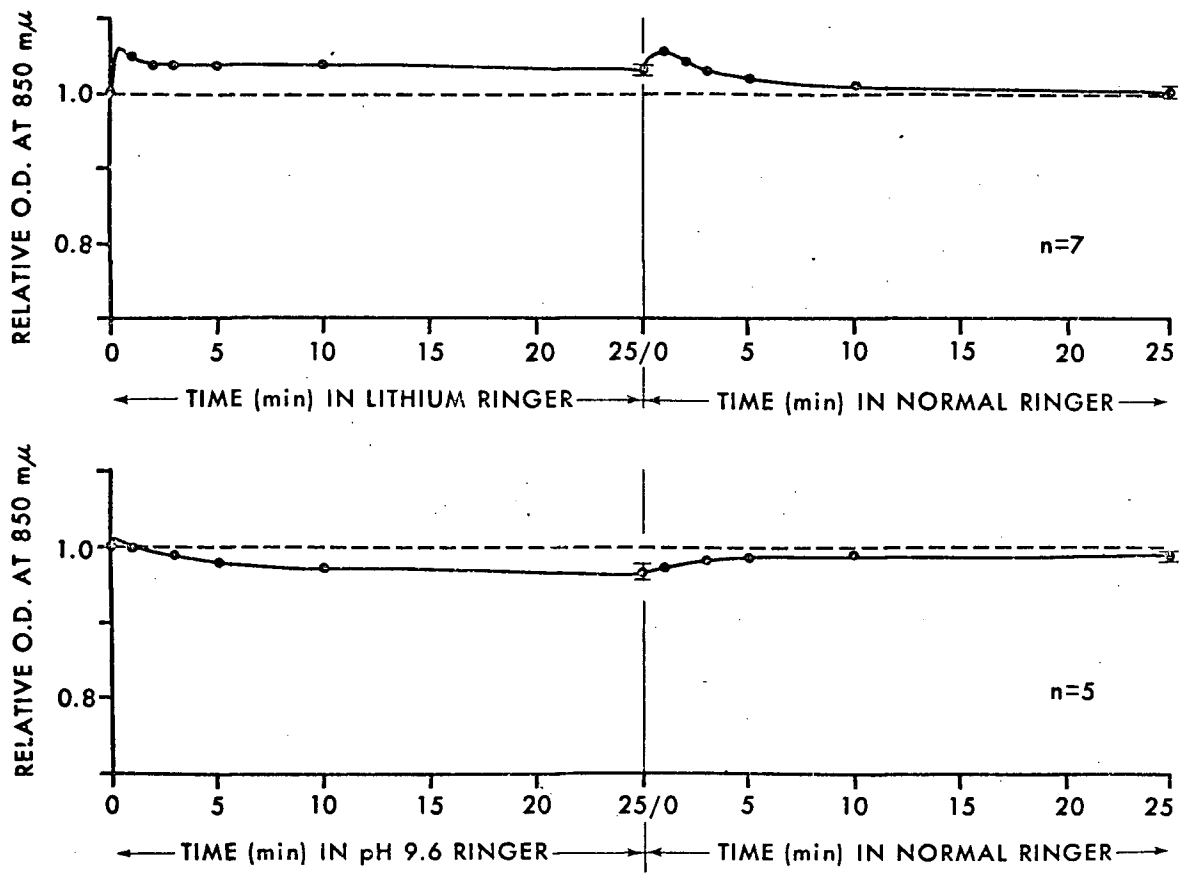

Fig. 16. The relative O.D. (850 mμ) of single muscle fibers bathed in lithium Ringer (upper graph) and in pH = 9.6 Ringer (lower graph).

(at 850 mμ) of single muscle fibers bathed in sodium free, lithium substituted Ringer is graphed as a function of time. No decrease in the O.D. was observed. In fact, a slight, but statistically significant increase occurred, which was reversible. After 25 minutes in lithium Ringer, the relative O.D. increased to 1.033 ± .003 (n=7) and upon returning normal Ringer to the bathing chamber the relative O.D. decreased to 1.004 ± .008 (n=7).

The changes in the total concentrations of potassium, sodium and lithium in single muscle fibers bathed in lithium Ringer are illustrated in

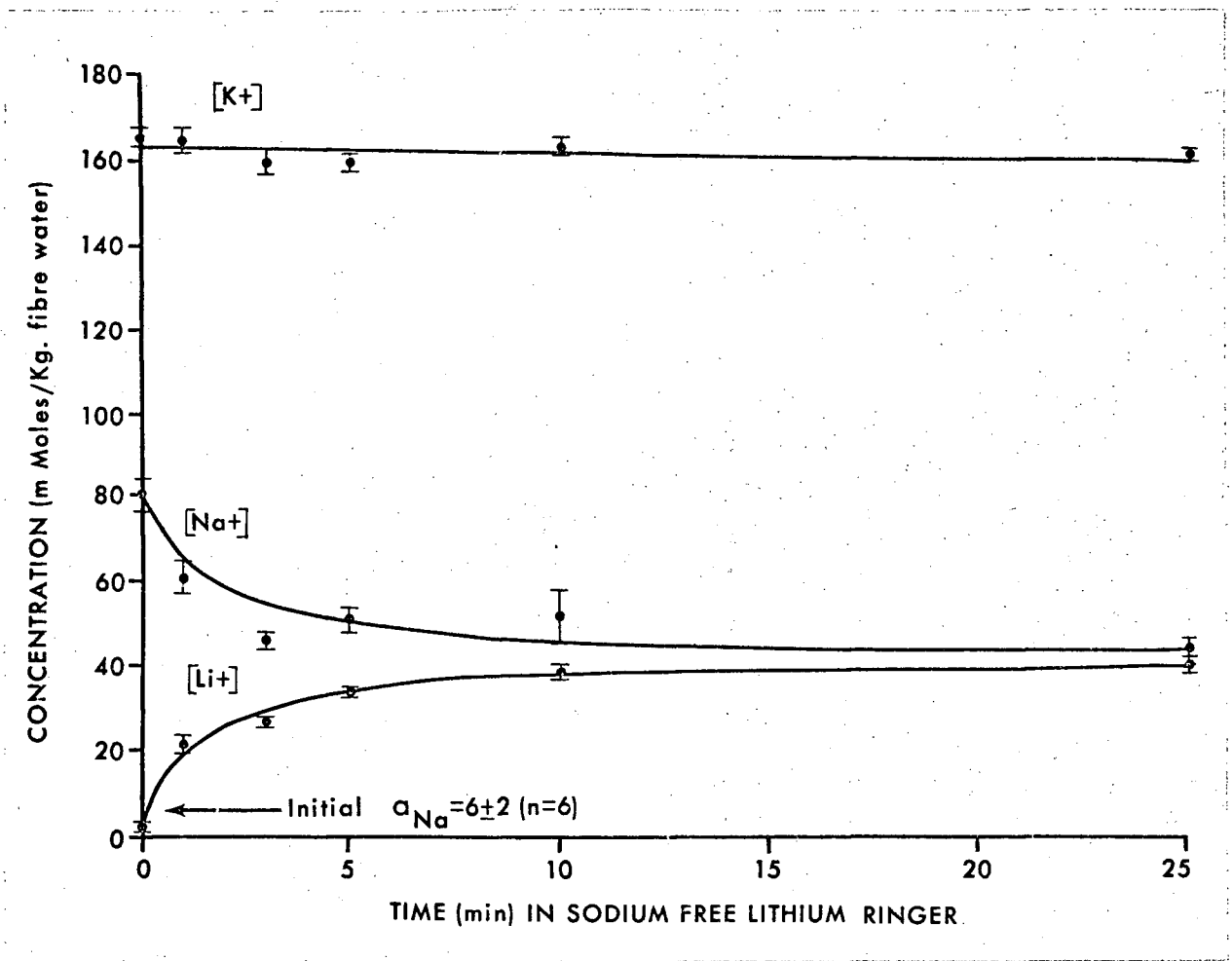

Fig. 17. The total concentrations of potassium, sodium and lithium in fibers bathed in sodium free, lithium substituted Ringer. Initial points are the average of measurements from 20 fibers. Other points are the average of measurements from 10 fibers.

Fig. 17. The concentration of potassium in the fibers remained constant (Fig. 17) as in fibers bathed in sucrose Ringer (Fig. 11). After 25 minutes in lithium Ringer the sodium concentration decreased by .080 - .044 = .036 moles/kg fiber water and the lithium concentration increased by 0.41 - .002 = .039 moles/kg fiber water (Fig. 17). For comparison, it should be recalled that the decrease in the sodium concentration after 25 minutes in sucrose Ringer was .066 - .035 = .031 moles/kg fiber water (Fig. 11).

One would like to know how much of the decrease in total sodium concentration of fibers soaked in lithium or sucrose Ringer was due to sodium leaving the extracellular space and how much was due to sodium actually leaving the cell. As mentioned above, the extracellular space comprises about 5% of the volume of a single muscle fiber, hence probably contains .030 moles/kg fiber water of sodium. Thus, in lithium Ringer, about .006 moles/kg fiber water of intracellular sodium leaves the cell and about .009 moles/kg fiber water of lithium enters the cell through the sarcolemma. These differences are not significant. In sucrose Ringer about .001 moles/kg fiber water of sodium leaves the cell in 25 minutes. It should be stressed that the extracellular space may have been slightly overestimated (6) and that it was not measured on the experimental fibers. Thus, a definite value cannot be assigned to the concentration of intracellular sodium lost in either sucrose or tris Ringer. A reasonable estimate would appear to lie between .005 and .010 moles/kg fiber water. The concentration of free intracellular sodium lost in either sucrose or tris Ringer is approximately identical after 25 minutes (Fig. 18) and equal to about .002 moles/kg fiber water. When this value is subtracted from the estimate of the total loss of intracellular sodium, it is apparent that only about .005 moles/kg fiber water of "bound" sodium was removed by the sucrose or lithium Ringer.

As mentioned above, the activity of sodium in the myoplasm, a_{Na}, of six fibers soaked in lithium Ringer was measured to determine the loss of the free intracellular sodium in this solution. Measurements were made with the same microelectrode used in the sucrose Ringer experiments (Fig. 11). Furthermore, measurements were made on alternate fibers from the same barnacles; one fiber was bathed in sucrose Ringer, the next in lithium

Ringer and so on. In lithium Ringer (see Fig. 17 for the initial a_{Na}) a rather unexpected, transitory increase in a_{Na} occurred in four out of six fibers, whereas in sucrose Ringer, a_{Na} always decreased monotonically with time. The sodium sensitive microelectrode was slightly sensitive to lithium, but the electrode readings were corrected for this by assuming that the activity of lithium in the myoplasm was equal to the total concentration of lithium in the fiber. Obviously this is a maximal correction, because most of the fiber lithium should be in the extracellular space.

The transitory increase noted in the a_{Na} of fibers bathed in lithium Ringer warranted the construction of an electrode which had no measurable response to lithium in the concentration range that could have occurred in the myoplasm and the repetition of the above experiments. Measurements were made with this electrode on 5 fibers bathed in lithium Ringer and on 4 fibers bathed in sucrose Ringer. The results were similar to those obtained previously. In lithium Ringer a transitory increase in a_{Na} occurred in 2 out of 5 fibers whereas in sucrose Ringer a_{Na} always decreased monotonically with time. The results are summarized in Fig. 18, which is a graph of a_{Na} as a function of time in lithium Ringer (upper curve) and in sucrose Ringer (lower curve) relative to the initial value of a_{Na} when the fiber is in normal Ringer. The differences between the two curves are only statistically significant for the first 5 minutes. Differences in the rate of decrease of a_{Na} could have resulted from variations in the size of the fiber, the position of the microelectrode in the fiber, the activity of the "sodium pump" or the initial a_{Na}, but it is difficult to conceive how any of these factors could have caused a_{Na} to increase in lithium Ringer. It seems likely, therefore, that the increase in a_{Na} reflects a release of sodium from an internal binding site.

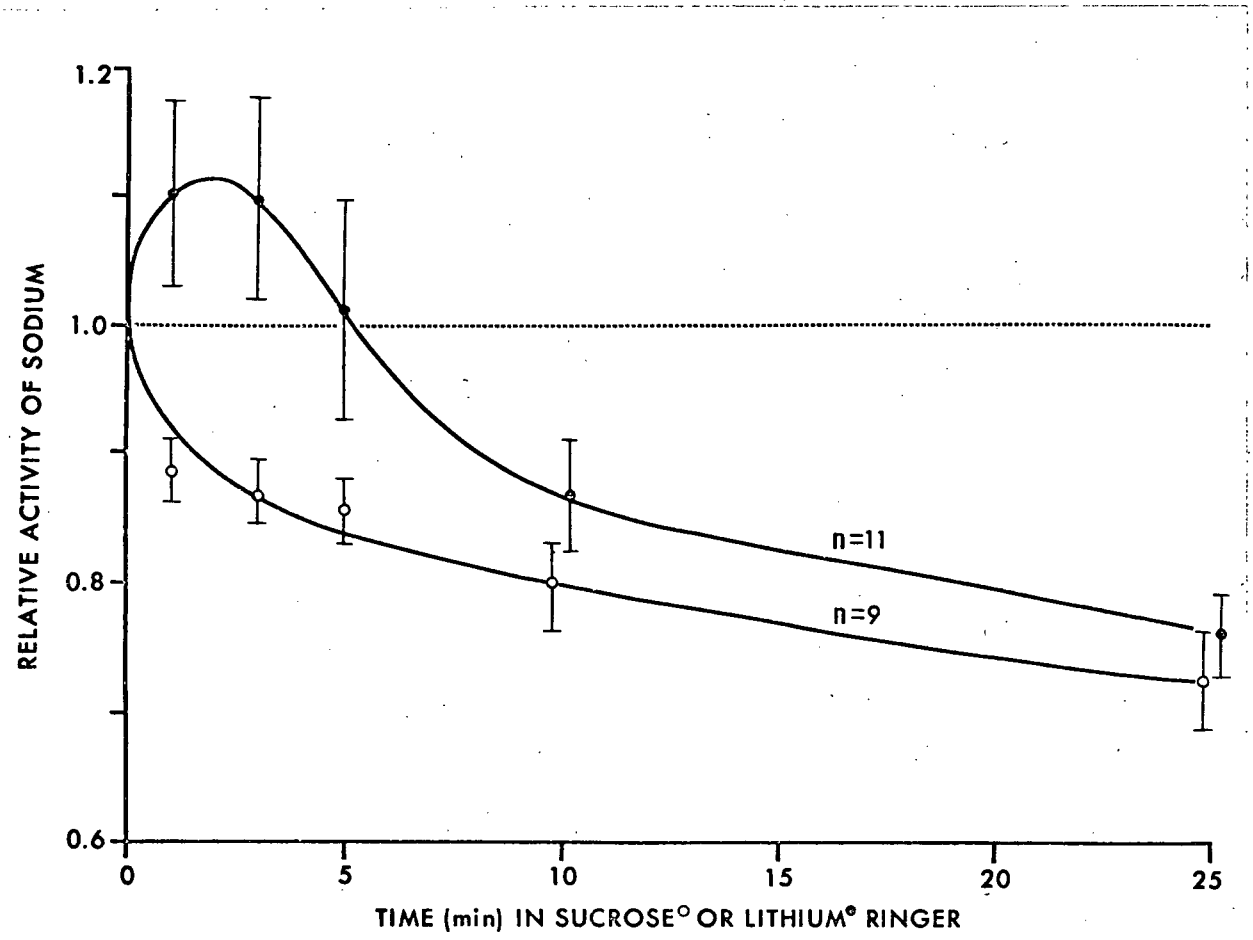

Fig. 18. The activity of sodium in the myoplasm, a_{Na}, of single muscle fibers bathed in lithium Ringer (upper curve) or sucrose Ringer (lower curve) relative to the initial a_{Na} when the fiber was bathed in normal Ringer. The initial a_{Na} of the fibers bathed in lithium Ringer was .006 ± .001 M. The initial a_{Na} of the fibers bathed in sucrose Ringer was .009 ± .002 M.

pH = 9.6 and pH = 5.5 Ringer. A simple experiment which illustrates the dependence of the O.D. of a muscle fiber on the charge of the macromolecules it contains was performed on glycerinated fibers. Fibers which had been bathed in .01 M KCl were placed in .01 M KOH and observed under a dissecting microscope. Within 10 minutes they became almost transparent. The change in O.D. was reversible. When the fiber was returned to normal Ringer, the O.D. of the fibers increased. The decrease in O.D. was

presumably due to the fact that the macromolecules in the fiber acquired a large net negative charge when it was bathed in .01 M KOH. Similar large reversible changes in O.D. were observed when glycerinated fibers were bathed in .01 M HCl. This decrease in O.D. was presumably due to the fact that the macromolecules acquired a large net positive charge in .01 M HCl.

The O.D. of a muscle fiber will be a maximum when the pH of the myoplasm is near the isoelectric pH of the main scattering centers in the fiber, which are presumably the thick filaments. It is essential for the argument advanced below that the thick filaments in a muscle fiber bathed in normal Ringer have a net negative charge. This assumption was tested by varying the pH of the myoplasm slightly. Increasing the pH of the myoplasm should increase the net negative charge on the thick filaments, hence decrease the O.D. of the fiber. Decreasing the pH of the myoplasm up to, but not beyond the iscelectric point of the thick filaments should decrease their net negative charge, hence increase the O.D. of the fiber.

The pH and membrane potential measurements made on four fibers bathed in pH = 9.6 Ringer are illustrated in Fig. 19. The pH of the myoplasm when the fibers were in normal Ringer was 7.315 ± .009 (n=4). After 25 minutes in pH = 9.6 Ringer it increased to 7.378 ± .018 (n=4). The O.D. changes that occurred in pH = 9.6 Ringer are illustrated in Fig. 16 (lower graph). The relative O.D. decreased to .966 ± .011 after 25 minutes.

Caldwell (7) has shown that the myoplasmic pH of crab muscle fibers may be rapidly and reversibly decreased by bathing the fibers in Ringer acidified with CO_2. A similar reversible decrease in the myoplasmic pH of barnacle muscle fibers bathed in Ringer which had been acidified with CO_2 (pH of Ringer = 5.5) was observed. The myoplasmic pH decreased from 7.3

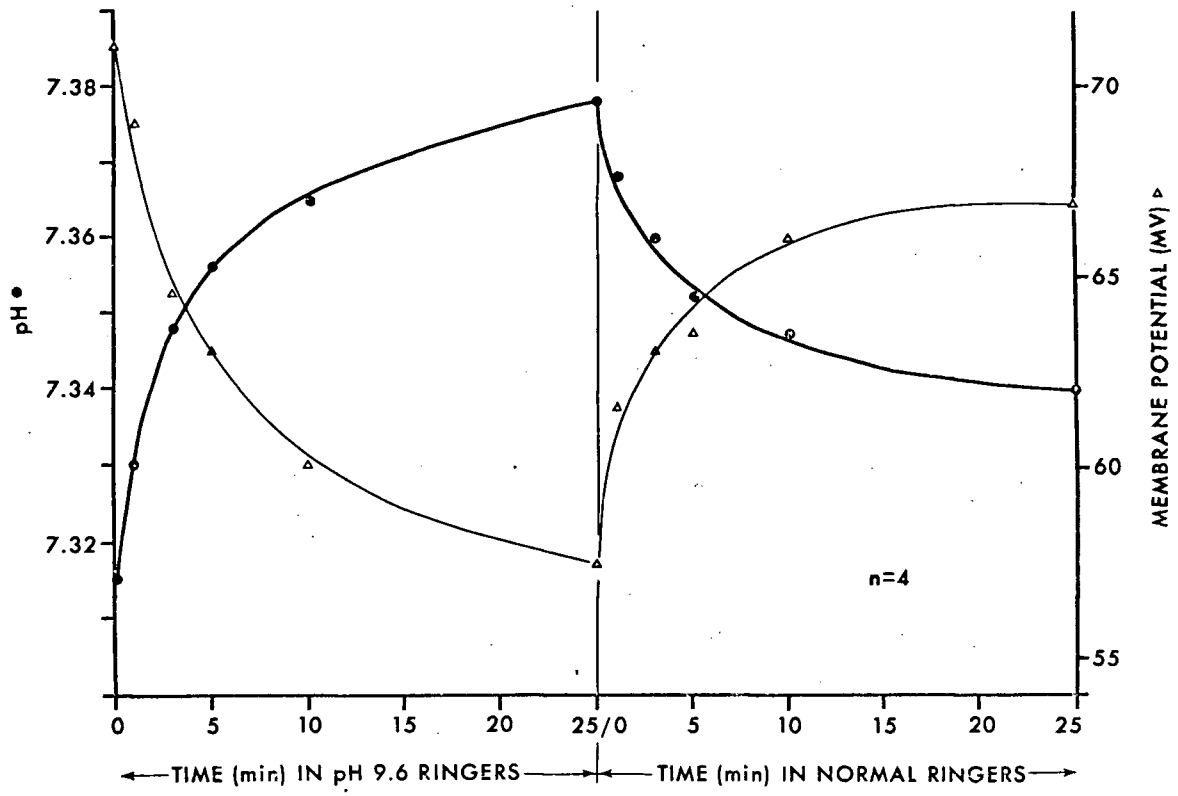

Fig. 19. The pH and membrane potential of single muscle fibers bathed in pH = 9.6 Ringer and in normal Ringer.

to 6.3 in 3 minutes. When normal Ringer (pH = 7.6) was returned to the bathing chamber, the pH of the myoplasm returned to 7.3 within 10 minutes (6). The relative O.D. of fibers bathed in pH = 5.5 Ringer increased to a stable value of 1.047 ± .006 (n=7) within three minutes. After 5 minutes in the pH = 5.5 Ringer, normal Ringer was returned to the bathing chamber. After 10 minutes in normal Ringer, the relative O.D. decreased to 1.017 ± .004 (n=7).

D. Discussion

The optical studies reported in this paper were undertaken to obtain independent supporting evidence for the hypothesis that sodium is bound to myosin in striated muscle fibers. Eqn. [27] illustrates the relationship that exists between the net charge, Z, on a small, optically inactive molecule and the turbidity, τ, of a solution when the concentration of the macromolecule is low and the ionic strength of the solution is high. For several reasons, neither Eqn. [27], nor more expanded and complete forms of it (3, 8) are quantitatively applicable to a muscle fiber. A fiber contains not one, but many macromolecular species capable of scattering light. The thick filaments, however, are probably the main scattering centers because of their high "molecular" weight and concentration. These filaments form a highly concentrated solution or gel, are large compared with the wavelength of incident light, and contain optically active molecules. These complicating factors make the quantitative application of light scattering theory exceedingly difficult, but they should not destroy the qualitative relation between macromolecular charge and turbidity.

A more serious complication arises from the fact that the thick filaments in a muscle fiber are not free in solution, but organized in a parallel hexagonal array. Increasing the net charge on macromolecules free in solution decreases the randomness of the solution, or equivalently, decreases the concentration fluctuations of the molecules, hence decreases the turbidity of the system. Increasing the net charge on macromolecules which are initially ordered will not necessarily decrease the concentration fluctuations. This will only occur if the initial order in the system is one for which the electrostatic free energy is a minimum. Fortunately, the parallel hexagonal array of the thick filaments is exactly the minimum electrostatic free energy configuration for a system of charged rods (9, page 233). Thus,

increasing the net charge on the thick filaments should decrease the magnitude of the concentration fluctuations of the filaments, hence decrease the turbidity of the muscle fiber.

It is also essential for the argument that the thick filaments be negatively charged. Titration (10), electrophoresis (11, 12) and ATP binding studies (11, 13, 14) on myosin indicate that this requirement is satisfied at the myoplasmic pH of about 7.3 (Fig. 19). The experiments in pH = 5.5 and pH = 9.6 Ringer also indicate the thick filaments are negatively charged.

In spite of the inherent difficulties in the application of light scattering theory to living muscle fibers, it seems reasonable to attempt to explain the optical results in terms of changes in the net charge on the thick filaments. The significance of the exercise lies in comparing the experimental results with the results predicted from the hypothesis that a significant fraction of sodium in the fiber is bound to the thick filaments, presumably to carboxyl moieties. When the fibers are bathed in sucrose, tris or potassium Ringer, sodium should move off the binding sites and out of the cell, causing an increase in the net negative charge on the thick filaments. This should decrease the turbidity, hence the O.D. of the fibers. Large, reversible decreases in the O.D. of fibers bathed in these solutions (Figs. 13, 14, 15) were indeed observed. The arguments advanced in Chapter II indicate that the binding sites on the thick filaments should prefer the alkali metal cations in the order Li>Na>K (15). Thus, when the fibers are bathed in lithium Ringer, the lithium entering the myoplasm should more than compensate for the loss of the "bound" sodium. This should cause a slight decrease in the net negative charge on the thick filaments, hence

the turbidity and the O.D. of the fiber should increase. A small reversible increase in the O.D. of fibers bathed in lithium Ringer was observed (Fig. 16). Furthermore, there was an initial increase of a_{Na} in fibers bathed in lithium Ringer (Fig. 18) which may also be due to the displacement of bound sodium by lithium. It should be emphasized, however, that these increases in a_{Na} were small, and observed only in about 50% of the fibers bathed in lithium Ringer. It is apparent then, that all the optical results are compatible with the hypothesis that sodium is bound to the thick filaments.

Unfortunately, a prediction of how much sodium is bound to the thick filaments cannot be made from these experiments. The optical changes appear to be due to the movement of only about .005 moles/kg fiber water of sodium from the binding sites, but there could be either more or less than this amount of sodium bound to the thick filaments. It should also be noted that other interpretations of the experimental results are possible, for the tacit assumption has been made that the structure of the thick filaments did not change when the fibers were bathed in sodium free solutions. The possibility, however, that sodium or lithium is necessary to maintain the integrity of certain structures in the myoplasm is itself extremely interesting. Obviously, much more experimental work remains to be done on the relationship between the optical properties of muscle fibers and the ions they contain, but at present these experiments are offered as qualitative support for the hypothesis that a significant fraction of the sodium in intact barnacle muscle fibers is bound to myosin.

CHAPTER VII

BINDING OF SODIUM AND POTASSIUM IN GLYCEROL EXTRACTED FIBERS

A. Introduction

The experiments performed by Lewis and Saroff (1) on solutions of extracted myosin indicate that this protein has the capacity to bind large quantities (about 50 moles/10^5 grams myosin) of alkali metal cations and that the association constant for the myosin-sodium complex is about twice the association constant for the myosin-potassium complex. It was noted in Chapter II, however, that one should be hesitant about using these results to predict quantitatively how much sodium and potassium are bound to myosin in a living cell. The spatial fixation and cross-linkage that myosin undergoes in the living cell may alter its binding characteristics, as experiments on other macromolecules and polyelectrolytes have indicated. It seemed reasonable, therefore, to study the influence of these two phenomenon on the capacity of myosin to bind sodium and potassium by measuring the binding characteristics of the contractile proteins in a glycerinated fiber. As the membranes in a fiber are destroyed by glycerination, and no source of metabolic energy remains, the possibility that ions are selectively accumulated in intracellular organelles may be ignored.

When the experiments in this chapter were undertaken, the author was unaware of the work of Fenn (2). Fenn had already demonstrated that glycerinated fibers selectively accumulate sodium over potassium when exposed to solutions containing equal concentrations of these two ions. The free concentrations (or activities) of sodium and potassium in the myoplasm of an intact striated muscle fiber, however, are far from equal, as the

measurements reported in Chapters IV, V and VI demonstrate. The selectivity of many ion exchange resins depends on the relative concentrations of the alkali metal cations in the exchanger (3). Thus, if the glycerinated fiber is to be considered a model, albeit a crude model, of the living cell, it seemed appropriate to study the accumulation of sodium and potassium by glycerinated fibers exposed to solutions containing these ions at approximately the same activities as are found in the myoplasm of an intact fiber. The results of such a study are presented below. They are qualitatively compatible with the results of both Lewis and Saroff (1) and Fenn (2) in that they demonstrate the selectivity coefficient, $K_{Na/K}$, of the glycerinated fiber is greater than 1.0 (see Eqn. [7] for a definition of the selectivity coefficient).

B. Methods

Glycerination. Approximately a dozen fibers on a single baseplate were dissected free from one another in normal Ringer (Table I) at 25° C and examined for damage. Only undamaged fibers were used. The tendons of these fibers were ligated by thread to a rod and the baseplate tied to another rod so that the fibers were fixed at rest length. The fibers were then transferred to glycerol (50% by volume, buffered to pH 7.33 at 25° C). The temperature of the glycerol was 2-3° C, and the fibers were stored for 20 hours at this temperature. The fibers were then transferred to fresh glycerol and stored at -20° C for 24 days. This glycerination procedure is similar to that used by Szent-Györgyi (4).

Test of Contractility. At least one fiber from each baseplate was tested for contractility before experiments were performed on the other fibers. The fiber was cut from the stone, bathed for 10 minutes in distilled

water to remove most of the glycerol, then placed in a solution containing .005 M ATP, .010 M $CaCl_2$, .010 M $MgCl_2$, .200 M tris at a pH of 7.6. All fibers tested for contractility did shorten. The contractions occurred slowly over a period of about 5 minutes. the fibers shortening to about 1/2 their initial length.

Experimental Procedure. All experiments were performed in a cold room at a temperature of 2-3° C. The fibers were first transferred from the glycerol solution to a solution containing .275 M KCl and .025 M KH_2PO_4 at pH 7.4. They remained in this solution for 1 hour to allow the glycerol to diffuse out of the fibers. They were then transferred to a solution containing .275 M KCl, .025 M KH_2PO_4 and either a trace (.0002 M) concentration or .010 M $Na^{22}Cl$ at pH 7.4. The fibers were equilibrated in one of these two solutions for 1-2 hours. After this period of time the fibers were cut from the baseplate, blotted briefly to remove excess bathing fluid and placed in preweighed, stoppered weighing bottles. The blotting and transfer procedure took about 10 seconds. The wet weight of the fiber was measured immediately, the fiber dried to a constant weight at 95° C, then the dry weight of the fiber measured. After digestion in .2 ml of concentrated HNO_3 and neutralization with ammonia, the liquid was transferred to a vial used in the Nuclear Chicago Automatic Gamma Well Counting System and diluted to 5 ml.

The sodium content, in counts per minute (cpm), of the experimental fiber was then measured. These measurements were alternated with measurements of the cpm/ml of the bathing solution. Eight one ml samples of the bathing solution were pipetted into vials (samples were also weighed on a six place balance to correct for any pipetting errors), diluted to 5 ml, and

the cpm determined. Samples were counted for 10 minutes, and 5 repetitions of the counts were made to ensure the stability of the gamma counter. The background was less than 1% of the cpm in the experimental samples.

After the radioactivity of the samples was determined, they were diluted to 25 ml and analyzed for potassium content on a Unicam SP 900 flame spectrophotometer. Samples of the bathing solution were also analyzed for potassium content.

C. Results

A preliminary experiment was conducted to determine how long the fibers should be equilibrated in the solutions containing Na^{22} before they were removed for analysis. Sixteen glycerol extracted fibers were bathed for one hour in a .275 M KCl plus .025 M KH_2PO_4 solution to remove the glycerol, then transferred to a similar solution containing Na^{22}. They were removed from this solution at times ranging from 1/2 to 18 hours. The sodium and potassium concentrations in the muscle reached a constant value after 1/2 hour. To ensure equilibration in the experimental series, fibers were bathed in the solutions containing Na^{22} for 1-2 hours.

The results of the experiments are presented in Tables VI and VII. The first column in Table VI gives the % water (by weight) of the fibers. The average value is 88.5%. The average % water content of an intact barnacle muscle fiber, on the other hand, is about 75% (average from the experimental fibers utilized in the experiments described in Chapters V and VI). This implies that the glycerol extracted barnacle muscle fibers have swelled to about twice their initial volume. The next column lists the dry weight of the fibers.

TABLE VI

The sodium and potassium content of fibers extracted in 50% glycerol for 24 days then equilibrated in a solution containing [K] = 295 mM and [Na] = 10.4 mM. The radioactivity in the bathing solution due to the Na^{22} was 23,530 ± 540 (n = 8) cpm/ml.

% Water in Fiber	Dry Weight 10^{-3}gms	[Na] cpm/fiber Measured	[Na] cpm/fiber Expected	[K] mMoles/fiber Measured x 10^{-3}	[K] mMoles/fiber Expected x 10^{-3}	Na bound cpm/gm dry weight	K bound mMoles/gm dry weight
84.1	2.649	397	330	4.500	4.133	25,300	0.138
88.6	1.925	416	352	4.750	4.413	33,200	0.175
89.7	1.940	453	397	5.375	4.984	28,900	0.201
88.1	2.428	452	423	5.500	5.303	11,900	0.081
88.2	1.874	393	329	4.500	4.132	34,200	0.196
88.9	3.358	803	633	8.875	7.934	50,600	0.280
90.6	3.150	965	714	9.250	8.956	79,700	0.093
88.8	2.390	539	446	6.000	5.590	38,900	0.171
88.9	2.090	599	394	5.375	4.938	98,100	0.209
88.5	2.568	617	465	6.250	5.830	59,200	0.164
90.1	2.103	666	450	6.000	5.646	102,700	0.168
88.5	2.453	615	444	5.875	5.569	69,700	0.125
87.6	2.396	459	398	5.375	4.993	35,500	0.159
88.5						51,400 ± 7,900*	0.166 ± 0.014

* S. E.

The third column lists the measured radioactivity of the individual fibers in cpm. The fourth column lists the values of the cpm expected on the basis of three assumptions. These assumptions are: (i) no sodium ions are bound to the contractile proteins, (ii) the activity coefficient of sodium ions in the glycerinated fiber is equal to the activity coefficient of sodium ions in the bathing solution, (iii) all the water in the cell is free to act as solvent for the sodium ions. The fourth column was calculated by multiplying the weight of water in the fiber by the measured value of 23,530 ± 540 (n=8) cpm/ml bathing solution, the radioactivity of the bathing solution. (The error of less than 1% that arises in assuming that 1

gm of water contains the same number of water molecules as 1 ml of bathing solution is ignored.) Note that in each case the fiber contains more sodium than would be expected on the basis of the above three assumptions. Assumption (iii) is probably not valid, but the effect of any water "binding" would be to reduce the concentration of sodium in the fiber, hence the invalidity of this assumption could not lead to the observed results. It will be assumed that assumption (ii) is valid, and that the observed accumulation of sodium by the glycerinated fibers is due to the binding of sodium to the contractile proteins.

The penultimate column lists the amount of sodium bound (in cpm) per gram of dry weight of muscle. The results in this column were obtained by subtracting the measured and the expected cpm and dividing by the dry weight of the fiber. The average value of 51,400 ± 7,900 (n=13) cpm/gm dry weight is equivalent to (51,400 cpm/gm dry weight)(0.0104 mMoles/23,530 cpm) = 0.023 mMoles bound sodium/gm dry weight.

The fifth column in Table VI lists the measured amount of potassium in the muscle, as determined by flame photometry. The sixth column lists the mMoles of potassium expected in the fiber on the basis of assumptions (i, ii, iii) applied to potassium instead of sodium ions. The final column lists the mMoles of bound potassium per gm of dry fiber weight. The average of this quantity is 0.166 mMoles/gm dry weight.

The glycerinated fibers could be considered as highly hydrated ion exchange resins, and the selectivity, as defined in Eqn. [7] of these resins calculated. The average selectivity of the fibers, calculated from the data of Table VI is $K_{Na/K} = 1.18$. There is, however, a growing body of evidence indicating that "we may, to a first approximation, regard the

TABLE VII

The sodium and potassium content of fibers extracted in 50% glycerol for 24 days then equilibrated in a solution containing [K] = 295 mM and [Na] = 0.2 mM. The radioactivity in the bathing solution due to the Na^{22} was 22,550 ± 630 (n = 8) cpm/ml.

% Water in Fiber	Dry Weight 10^{-3}	[Na] cpm/fiber		[K] mMoles/fiber		Na bound cpm/gm dry weight	K bound mMoles/ gm dry weight
		Measured	Expected	Measured x 10^{-3}	Expected x 10^{-3}		
87.3	2.463	415	382	5.375	5.000	13,400	0.152
90.1	3.164	716	649	8.500	8.495	21,200	0.002
90.2	2.775	690	576	7.875	7.535	41,100	0.122
88.0	2.936	581	485	6.625	6.352	32,700	0.093
90.2	2.214	623	459	6.250	6.012	74,100	0.107
90.4	2.816	665	598	7.875	7.823	23,800	0.018
89.8	2.458	602	488	6.500	6.384	46,400	0.047
88.4	1.230	249	211	3.250	2.765	30,900	0.394
88.3	3.298	763	561	7.750	7.343	61,200	0.123
86.9	2.559	468	383	5.375	5.008	33,200	0.143
90.6	3.813	920	828	11.625	10.842	24,100	0.205
87.1	2.878	555	438	6.125	5.732	40,700	0.136
87.6	2.542	473	404	5.625	5.298	27,100	0.128
88.9	2.360	555	426	6.000	5.576	54,700	0.180
88.8						37,500 ± 4,500*	0.132 ±0.025

* S. E.

effect of addition water as merely to 'dilute' the processes giving rise to selectivity" (5). Thus, it seems reasonable to consider not only the selectivity of the fiber as a whole, but the selectivity of the proteins as well. The term $K_{Na/K}^{\lim H_2O \to 0}$ may be defined as (mMoles of sodium bound per gm protein/mMoles sodium free per ml solution)/(mMoles of potassium bound per gm protein/mMoles potassium free per ml solution). It may be calculated from the data of Table VI that the selectivity of the proteins, defined in this manner is (51,400/23,530)/(0.166/0.295) = 3.88.

The results of another series of experiments conducted in a bathing solution containing only a trace (0.2mM) concentration of sodium are

presented in Table VII. The selectivity coefficient of the proteins, $K_{Na/K}^{lim\ H_2O \rightarrow 0}$, calculated from the data in this table is (37,500/22,550)/(0.132/0.295) = 3.72, not significantly different from the selectivity coefficient calculated from the data of Table VI.

D. Discussion

Some of the factors which complicate the interpretation of the above measurements will now be discussed. At a pH of about 7.4, the proteins myosin and actin are negatavely charged (4) and the conditions for a Donnan equilibrium are established. The Donnan effect could not explain the selective accumulation of sodium over potassium by the fibers, but it could account for the potassium and some of the sodium accumulation. If the potassium accumulation of the muscle fibers was due to the Donnan effect, the fibers should have a negative potential $\Delta E = \frac{RT}{F} \ln ([K]solution/[K]fiber)$. From Table I, the average value of [K]solution/[K]fiber is 1.073, hence the fiber should have a negative potential of about 1.8 mV. The measured potential of the muscle fibers was 0.00 ± 0.05 mV, indicating that if the muscle fiber is considered as a homogeneous entity, the Donnan effect plays a negligible role in the accumulation of ions. The author feels, however, that one should no longer consider either an intact or a glycerinated muscle fiber as a homogeneous entity. Rather, one should note that there are at least "two types of electrostatic binding, which differ in the degree of specificity of binding between charged groups. One of these might be characterized as ion-pair formation and the other as a generalized domain binding with the small counterions associated as a mobile layer with the large, multiply charged molecule." (6) The selectivity of the binding argues for the former interpretation, but does not prove it.

The possibility that the proteins could affect the activity coefficients of sodium and potassium ions without actually "binding" these ions should be considered, but lack of information about this phenomenon necessitates the assumption that the activity coefficients of the "free" ions are unaffected by the proteins.

Also worthy of consideration is the possibility that a fraction of the water in the glycerol extracted fibers is "bound" in such a manner that it is unavailable to act as solvent for the alkali metal cations. A method of measuring experimentally this fraction of water is discussed in Chapter IX.

It may be worthwhile to summarize the available information about the binding of sodium and potassium to extracted muscle proteins. The experiments of Lewis and Saroff (1) indicate that a maximum of 0.50 mMoles of cations can be bound to 1 gm of myosin, and that myosin binds sodium more strongly than potassium. The electrophoresis experiments of Miller et al (7) also indicate the sodium is bound more strongly than potassium to myosin. In an abstract published in 1942, Mullins (8) reported that myosin preferred potassium to sodium, but Fenn, who repeated these experiments, concluded that "the published abstract was in error for unknown reasons" (2).

Both the magnitude and the selectivity of the binding that Fenn (2) observed in glycerol extracted fibers are compatible with the results of Lewis and Saroff (1). If it is assumed that the glycerol extracted fibers used by Fenn contained 75% water by weight, and further assumed that the concentrations he quotes in meq/kg refer to a kg of muscle and not fiber water, one may calculate the limiting selectivity from the data presented in

Table II of his paper. The average value of $K_{Na/K}^{lim\ H_2O \to 0} = 2.8 \pm .3$. This selectivity does not appear to be dependent on the concentration of sodium and potassium in the bathing solution.

In summary, a glycerinated fiber can be considered an ion exchange resin and the selectivity of the "resin", $K_{Na/K}$, calculated. Fenn's experiments demonstrated the selectivity was greater than unity when the ions were present in equal concentrations: the experiments presented here illustrate it is also greater than unity when sodium and potassium ions are present at concentrations similar to those found in an intact fiber. The average selectivity of the proteins (not of the fiber as a whole) for the two experimental series was $K_{Na/K}^{lim\ H_2O \to 0} = 3.8$. If the glycerol extracted fiber is considered as a model of the intact fiber, the results of Table VI imply that an intact fiber (containing 75% water by weight) with $a_K = 0.20$ M and $a_{Na} = 0.007$ M contains 0.055 moles/kg water of potassium and 0.008 moles/kg water of sodium "bound" to proteins.

CHAPTER VIII

SIGNIFICANCE OF THE RESULTS

Probably the most important conclusion that can be drawn from the experiments presented in this thesis is that one can no longer consider the alkali metal cations and water in a striated muscle fiber to be in exactly the same state as the ions and water in the bathing solution surrounding the cell. Consider the sodium content of a single muscle fiber from the giant barnacle, Balanus nubilus. A typical intact fiber contains about 70 mMoles/kg fiber water of sodium (Chapters IV, V, VI). About 30 mMoles/kg fiber water of sodium are contained in a compartment which communicates with the bathing solution; presumably the extensive invaginations of the sarcolemma which are visible under the electron microscope (1). Only about 10 of the remaining 40 mMoles/kg fiber water of sodium are "free" in the myoplasm, as was determined directly by cation sensitive microelectrodes (Chapters IV, V, VI). Thus, about 3/4 of the intracellular sodium in barnacle muscle fibers is "bound". It seems reasonable to extrapolate this conclusion to all striated muscle fibers because Lev (2), who used cation sensitive microelectrodes to investigate the state of sodium in frog striated muscle fibers, obtained similar results and Robertson (3) found that 3/4 of the sodium in lobster muscle could not be extruded by pressure. Experiments performed with potassium sensitive microelectrodes indicate that between 27 and 41% of the intracellular water is unavailable to act as solvent for the potassium ions (Chapters IV, V). Thus, the microelectrode experiments reported in this thesis provide strong evidence that there is a hetrogeneous distribution of sodium and water in single striated muscle fibers from the giant barnacle.

The division of the sodium content of a striated muscle fiber into a free and a "bound" fraction is an oversimplification, but there is strong evidence that at least 1/3 of the "bound" sodium is complexed to myosin. Experiments on solutions of extracted myosin (4) and glycerinated fibers (Chapter VII) indicate that about 10 mMoles/kg fiber water of sodium may be expected to be complexed to myosin in an intact barnacle muscle fiber. This conclusion is fully supported by the denaturation and light scattering experiments reported in Chapters V and VI respectively.

The location of the remaining 20 mMoles/kg fiber water of sodium which is unavailable to a sodium sensitive microelectrode is unknown at present. It seems unlikely that this fraction of "bound" sodium is contained in nuclei or mitochondria, because these organelles comprise only a small fraction of the cell by volume, and do not appear to accumulate sodium preferentially over potassium (5, pages 226-229). The cisternae and longitudinal tubules of frog muscle have been estimated to comprise about 13% of the cell by volume (6), but the experiments of Zadunaisky (7) indicate that these compartments probably do not contain a high concentration of sodium. Furthermore, the sarcoplasmic reticulum appears to be less highly developed in barnacle than frog muscle. Thus, there is no evidence to indicate that this fraction of "bound" sodium is sequestered in organelles, but there is NMR evidence to indicate that it is bound to macromolecules (8). As discussed in Chapter IV, Cope's conclusion (8) that 3/4 of the sodium in striated muscle fibers is bound to macromolecules must be regarded as tentative at present, but this investigator knows of no experiment performed on intact striated muscle fibers which contradicts the conclusion. The experiments performed on glycerol extracted fibers (Chapter VII) do argue against this conclusion, but the selectivity of the binding sites for

sodium over potassium could be greater in the intact than in the glycerol extracted fiber.

What is the significance of these results? For one thing, they contradict the equilibrium or sorption theories of ionic accumulation put forward by individuals such as Nasonov (9), Troshin (10), Ling (5) and others. Ling (5), for example, contends that the carboxyl sites on proteins in the cytoplasm have a strong preference for potassium over sodium ions even though it has been known for over a decade that extracted myosin prefers sodium to potassium (4) and that glycerinated fibers preferentially accumulate sodium over potassium (11). It is the author's opinion that the experiments performed with cation sensitive microelectrodes on crab (12), frog (2) and barnacle (Chapters IV, V, VI) muscles directly disprove Ling's theory. The activity of potassium in the myoplasm of striated muscle fibers is not approximately equal to the activity of potassium in the bathing solution, as Ling's theory demands. It is the activity of sodium, not potassium, which has an anomalously low value.

Although the microelectrode experiments reported in this thesis directly contradict the equilibrium theories of ion accumulation, they also strengthen a criticism of the more generally accepted membrane theory of ion accumulation. This criticism, which has been strongly advanced by Ling (5, 13, 14), is concerned with the energy requirements of the postulated "membrane pumps". Aside from Ling, four groups of workers have studied this problem in relation to the postulated sodium pump in striated muscle fibers (15, 16, 17, 18). The consensus of opinion was that under physiological conditions about 20% of the total energy of the cell would be required to drive the sodium pump. This is a minimal value because both the

energy-delivering mechanism and the pumping mechanisms were assumed to be 100% efficient and it was further assumed that the diffusion of sodium in the myoplasm was surface rather than bulk phase limited. The direct measurements of the activity of sodium in the myoplasm indicate that the difference in the chemical potential of sodium across the sarcolemma is about 4 times the value calculated from measurements of the total concentration of sodium in the cell. Correction for this factor alone raises the energy requirements of the sodium pump in frog muscle from 20% to 25% of the total energy output of the cell. It is known that calcium and magnesium, as well as sodium ions, are permeable and not distributed across the sarcolemma according to the Nernst equation. Ling (14), using only the flux data available in the literature, calculated that the energy requirements of these three pumps is 330% of the total energy expenditure of the cell. Many other solutes such as hydrogen ions (Chapter VI), amino acids and sugars (14, 5) are also not distributed according to the Nernst equation, and presumably require energy expending "pumps" to maintain the disequilibrium.

This is a serious criticism of the membrane theory, but it does not imply that it must be rejected and an equilibrium theory of ion accumulation accepted in its place. Consider what is meant by the term active transport. It is usually defined as a process that can bring about a flow of a substance against an electrochemical potential gradient of the substance (19, 20). The existence of such a flow, however, does not mean that metabolic energy must be expended directly to cause the flow. As Katchalsky and Curran (21, page 199) point out "In principle, such flow could be anticipated on the basis of the thermodynamic equations without implying the operation of an active transport. A diffusional flow against its concentration gradient driven by dissipation of another diffusional process would be

regarded as an incongruent diffusion, not as active transport. Thus, the flow of the i^{th} component across a membrane may be written

$$J_i = L_{ii}\Delta\mu_i + \sum_{\substack{k=1 \\ k\neq i}}^{n} L_{ik}\Delta\mu_k \quad \text{.....[31]}$$

If $\Delta\mu_i = 0$, but $\Delta\mu_k \neq 0$, a flow of i may still take place." In Eqn. [31], L_{ij} represents the phenomenological coefficient which relates the difference in the electrochemical potential of the j^{th} species, $\Delta\mu_j$, to the flow of the i^{th} species, J_i. Spanner (22) also recognizes this possibility and gives an example of a hypothetical process which could bring about a flow of a substance against its electrochemical potential gradient, and furthermore be inhibited by metabolic poison, yet still not be driven directly by metabolic energy. Salminen et al (23, 24) have demonstrated experimentally that sodium and potassium ions may be made to flow in opposite directions against their concentration gradients across a synthetic membrane when there is a simultaneous flux of water and hydrogen ions in the system. A recent phenomenological description of the active transport of salt and water appears in a paper by Hoshiko and Lindley (25).

The above paragraph dealt with cases where a net flow of solute occurs against an electrochemical gradient, as in the intestinal mucosa, the wall of kidney tubules, frog skins and sodium loaded muscle fibers. The second law of thermodynamics dictates that such a flow must occur at the expense of energy, but it was noted that the energy need not be expended directly. (It is obvious that there can be no criticism of the ultimate metabolic energy expended in cases where a net transfer occurs. The cell must have sufficient energy to bring about such a transfer.) If a steady state system is now considered (for example, a resting muscle fiber) there is a complete range of energy the cell could expend to maintain this steady

state. If the steady state in fact represents an equilibrium, as Ling contends, no energy would be required. The author feels this possibility must be rejected, as stated above. The energy requirement of the cell (for a given set of fluxes and electrochemical potential gradients) is maximized if the steady state is maintained by a system of "pumps", each of which utilizes metabolic energy directly to pump sodium, calcium, magnesium and hydrogen ions against an electrochemical gradient and wastes the free energy gained by the cell due to the passive movement of these ions down their electrochemical gradients. It is the energy requirements of such a postulated system that Ling has repeatedly criticized (5, 13, 14). If Ling's calculation of the energy requirements of such a system of pumps is accepted, the concept of pumps which are uncoupled, or coupled only to another "uphill" process (such as the postulated sodium potassium coupling in muscle and nerve) must be rejected. Ling, however, is not logical in stating that "Unless we are willing to venture that the second law of thermodynamics does not hold in these living cells and that the living cells can generate free energy de novo, then within the confine of our understanding of the physical world there is no alternative to discarding the pump mechanism for selective ionic distribution in living cells" (14). The concepts of pumps need not be rejected at all. It need only be modified to accept the fact that coupling does occur between the various fluxes of solutes.*

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*The equations of irreversible thermodynamics would seem to imply that no coupling can occur when all the net fluxes are zero across a biological membrane (26, page 44), that is, when the cell is in a steady state at rest. This would be true if the net flux of an ion through each microscopic pathway in the membrane was zero. The postulation of a pump of any kind,

One possible coupling process is "exchange diffusion" (27); in such a process the influx of an ion would be directly coupled with the efflux. It is apparent that the existence of "exchange diffusion" would reduce the energy requirements of the sodium, calcium, magnesium, etc. pumps (for a given electrochemical potential gradient and flux). It should be stressed, however, that this is not the only possible mechanism whereby coupling can occur. Coupling could occur directly between the fluxes of two different ions in the membrane, via the current generated by a pump (recall the discussion in Chapter V about the possibility of an electrogenic pump) or via the production of an intermediate such as ATP. With regard to the last possibility, note that Garrahan and Glynn (28) have recently demonstrated that the influx of sodium ions in red blood cell ghosts can lead to the production of ATP. Further comment on the relationship between the transport processes and chemical reactions that could occur in biological membranes would be mere speculation, for as Eisenman notes (29), it is not even known how ions permeate through biological membranes.

A knowledge of the activities of sodium and potassium in the myoplasm is of value in other fields of membrane study such as the measurement of permeabilities and the evaluation of the membrane potential. To trans-

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however, is equivalent to postulating that the membrane is anisotropic (Curie-Prigogine Principle), and furthermore implies that through at least two microscopic pathways there exist non zero fluxes of the ion in question. The vector sum of all the component fluxes is of course equal to zero, but there is no difficulty in admitting that these individual fluxes are capable of being coupled.

form the flux rate of an ion into the permeability, for example, the difference in the activities of the ion across the sarcolemma must be known. In the past, investigators have assumed that this was equal to the difference in the concentrations of the ion across the sarcolemma. The measurements presented in Chapters IV, V and VI indicate that for both sodium and potassium ions, this assumption is erroneous.

CHAPTER IX

SUGGESTIONS FOR FUTURE WORK

A. Ion and Water Binding

Intact Fibers. The studies on carboxylic resins (1, 2) discussed in Chapter II indicate that these resins prefer the alkali metal cations in the order Li>Na>K. It is now known that both extracted myosin (3) and glycerol extracted fibers (4, Chapter VII) also prefer sodium to potassium and that the activity of sodium, but not potassium has an anomalously low value in the myoplasm of striated muscle fibers (Chapters IV, V, VI). It would be simple to determine if proportionally more lithium than sodium is "bound" in an intact muscle fiber. This experiment would support the tentative conclusion, based on the light scattering experiments reported in Chapter VI, that lithium is bound more strongly to myosin than either sodium or potassium, a conclusion compatible with the turbidity measurements of Szent-Györgyi (5, page 42) on solutions of extracted myosin in various concentrations of the alkali metal cations. Furthermore, the study would be of theoretical value in understanding the nature of the binding sites. There is no difficulty in exchanging most of the sodium in a barnacle muscle fiber for lithium. This can be accomplished by bathing the fibers in a sodium free, lithium substituted Ringer solution (preliminary experiments). There exist glasses, sufficiently sensitive to lithium (sodium being the only important contaminant), from which microelectrodes could probably be constructed (6). Thus, the activity and total concentration of lithium in the fiber could be measured, and the fraction of "bound" lithium determined.

Further information about the nature of the sites to which sodium

(and presumably potassium) ions are complexed with in intact muscle fibers could be obtained by measuring the activities of these ions in the myoplasm under conditions of varying pH. The pH of the myoplasm can rapidly be lowered by about 1 unit by exposing the muscle fiber to solutions saturated with CO_2 (Chapter VI). A few preliminary experiments indicated that the activity of sodium did not change significantly when the pH was lowered (7), but no measurements were made of the activity of potassium. It would be desirable to repeat, and extend these measurements to higher pH regions, if a method of rapidly increasing the pH of the myoplasm could be found.

The theoretical basis and experimental justification of using a diffusion technique to measure the fraction of ions bound to macromolecules in solution was given in Chapter II. Diffusion experiments have already yielded valuable information about the state of potassium in giant axons. The elegant experiments of Hodgkin and Keynes (8) demonstrated that the diffusion coefficient of potassium in giant axons is about 1.5×10^{-5} $cm^2 sec^{-1}$. The self diffusion coefficient of potassium in .5 M KCl is 2.135×10^{-5} $cm^2 sec^{-1}$ (9) and in .5 M KI $2.030\ 10^{-5}$ $cm^2 sec^{-1}$ (10). Thus, it appears that less than 25% of the potassium in giant axons is bound or compartmentalized. Caution, however, must be used in the interpretation of diffusion measurements made in biological material. Ling (11, page 338) points out that ion pair formation does not always decrease the diffusion coefficient of an ion. The diffusion coefficient of potassium on the surface of a glass, for example, is higher than that in a dilute solution (12). Presumably, this is because potassium can "jump" from site to site on the glass. It would be difficult, however, to argue that the anionic sites in a living cell are close enough to permit the movement of potassium by this type of mechanism. Even Ling has attempted to measure the diffusion

coefficient of ions in muscle as a demonstration of binding (13), but the diffusion experiments performed on muscle fibers to date have been far from conclusive (13, 14, 15). The extremely large barnacle muscle fibers would be ideal for measurements of the relative longitudinal diffusion coefficients of the alkali metal cations in the myoplasm. Two of these ions (lithium and radioactive sodium or potassium, for example) could be simultaneously injected into a fiber (16), the fiber sectioned after a given time, and the relative diffusion coefficients determined. The extensive membraneous network in these striated muscle fibers, however, would greatly impede the diffusion of both species, making accurate measurements extremely difficult. If it was found necessary to make kinetic measurements of the mobilities (as was done in the experiments of Hodgkin and Keynes), the experiments would have to be performed in a calcium free medium to prevent contraction of the fibers on application of a current. Thus, there are formidable technical difficulties to performing an experiment of this type, but the experiment would provide an excellent independent test of the microelectrode results presented in this thesis. Diffusion experiments on glycerinated fibers, on the other hand, would be relatively easy to perform and interpret.

Glycerinated Fibers. There are several advantages to studying ion and water "binding" on glycerinated rather than intact fibers. The pH and ionic concentrations may be rapidly and reversibly varied and the possibility of selective accumulation in intracellular organelles need not be considered. The destruction of the sarcolemma implies that the state of water in glycerinated fibers can be investigated by a variety of techniques not applicable to an intact fiber. One method would be to study desorption curves, as has been done on other non-membraneous tissues (17). Another would be to measure the imbibition pressure, as has been done on the corneal

stroma (18).

One could study simultaneously the binding of ions and water to the proteins in a glycerinated fiber by a simple technique. Consider Eqn. [19]. If both sides of this equation are divided by the weight of solid material in the glycerinated fiber, M_{pro}, the following equation results

$$B_K/M_{pro} = C_K V/M_{pro} - \alpha_K a_K V/(\gamma_K M_{pro}) \quad \text{.....[32]}$$

where B_K/M_{pro} represents the moles of potassium ions bound per kg of solid material. It is certainly reasonable to expect that B_K/M_{pro} will be a monotonically increasing function of a_K/γ_K (the free concentration of potassium in the muscle fiber, which is equal to the concentration of potassium in the external solution if the activity coefficients are identical), and approach a constant value when saturation of the binding sites occurs. This is indeed the case for myosin, as Fig. 1 of Lewis and Saroff (3) indicates. As their studies were performed at protein concentrations of less than 1% (3), their tacit assumption that the fraction of water available to act as solvent for potassium, α_K, was unity was probably valid. The value of α_K in a glycerinated fiber, however, may be significantly different from unity. If α_K is incorrectly assumed to be unity, B_K/M_{pro} will pass through a maximum, then fall instead of attaining a constant value. This indicates a method of measuring B_K/M_{pro} and α_K independently. A series of measurements at varying external concentrations of potassium could be fitted with a value of α_K which yields a horizontal line for B_K/M_{pro} at high concentrations. The experiments could be repeated with each of the alkali metal cations. This would indicate two things; the amount of water unavailable to act as solvent for each cation and the relative values of the association constants. The data of Fenn (4) indicates that a significant

amount of water "binding" will be found, for if his data are plotted in this manner the apparent binding (that is, the binding calculated with the assumption that α_K and α_{Na} = 1.0) of both sodium and potassium passes through a maximum at concentrations of about .10 M.

A series of measurements of the relative binding constants of the alkali metal cations to the proteins in a glycerinated fiber, coupled with more selectivity measurements of the type reported in Chapter VII would be of great theoretical value. They would allow one to examine critically a given theory of cation selectivity as applied to a biological fixed charge system. Two such theories, those of Eisenman (19) and Ling (11) were briefly discussed in Chapter II.

A critical examination of the theories of the binding of the alkali metal cations to proteins would be of value, but it would also be desirable to consider in more detail how proteins can affect the activity coefficients of ions without actually "binding" these ions. In Chapter IV it was noted that a knowledge of the macroscopic dielectric constant of a solution of proteins is of little value in predicting the effect of the protein on the activity coefficients of the ions in solution. Any theory of such effects should, as Edsall and Wyman (20) point out, be "framed in terms of the dimensions, dipole moments, and electrical polarizabilities of the individual molecules, rather than in terms of the macroscopic dielectric constant of the whole medium."

B. Denaturation and Contraction

The denaturation experiments reported in Chapter V could be extended to include an EM investigation of the changes in ultrastructure that

occur when a barnacle muscle fiber is heated to 37-40° C. It was postulated that the contraction and release of bound sodium and hydrogen ions that occurred at this temperature were due to a breakdown of the thick filaments. This conclusion was based on experiments performed on extracted proteins and glycerinated fibers. Either a confirmation or negation of this postulated breakdown of the thick filaments would be interesting, and could possibly shed some light on the mechanism of normal physiological contractions.

It was mentioned in Chapter V that some preliminary measurements were made to determine if the activities of sodium and potassium in the myoplasm varied when the muscle fiber contracted. The fiber was contracted by exposure to a solution containing 0.064 [K], and remained in a state of contracture for about 10 minutes. No significant change in a_K occurred during the isometric contracture (10 fibers). In 3 out of 9 fibers in which a_{Na} was measured, however, a large (100-500%) transitory (lasting about 30 seconds) increase in a_{Na} was noted at the start of contracture. The experiments were abandoned mainly because of two technical problems; the sodium electrodes were generally slow in responding to potential and activity changes and there was some doubt as to whether the observed potential change on the sodium sensitive microelectrode was due to an activity change or to a transitory depolarization of an internal system of membranes. These two difficulties could perhaps be circumvented by utilizing open tip cation sensitive microelectrodes and working with a depolarized preparation to which calcium could be added to produce contraction.

C. Light Scattering

The measurements reported in Chapter VI could be extended to study the effects of other ions on the O.D. of muscle fibers; cesium and rubidium

are obvious choices, but it would be interesting to study the effect of calcium and magnesium in more detail. It would also be desirable to make measurements of the angular as well as the wavelength dependence of the scattered light. This would require more elaborate instrumentation than a simple spectrophotometer, but would prove conclusively that the phenomenon was due to scattering and not absorption. Experimental measurements of the angular distribution of the scattered light could also be correlated with predictions made on the basis of the interference theory of light scattering because the size and distribution of the thick filaments in a muscle fiber are known from electron microscopy.

The investigator has also observed that during an isometric contracture (induced by an increase in the potassium concentration in the bathing solution) the O.D. of the muscle fibers increases markedly, then decreases on relaxation (preliminary experiments). This change may be due to the conjunction of the thick and thin filaments in the muscle fiber, an analogous phenomenon being the reversible increase in the O.D. of solutions of gelatin on setting (21). If further development of the theory of light scattering in muscle fibers indicates that the tentative explanation offered for the increase in O.D. is correct, this observation will be of fundamental physiological importance. Light scattering measurements may be made very rapidly ($<10^{-3}$ seconds) hence it would be possible to measure routinely on the same fiber: (i) the stimulating action potential with an open tip microelectrode, (ii) the conjunction of the thick and thin filaments by optical measurements, (iii) the contractile force with a transducer. As an example of the value of kinetic optical measurements in studying contractile phenomena, consider the recent experiments of Yefimov and Frank (22). They recognized the fact that the cross striations in a muscle fiber make it

analogous to a diffraction grid, and measured kinetically the changes in the grid constant that occurred on contraction. The results were extremely interesting; they found that shortening occurs not monotonically, but stepwise with time.

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APPENDIX I

Proof that a constant absorption decreases the experimentally observed changes in optical density. Let $O.D._1$ and τ_1 be respectively the optical density and turbidity of a fiber in normal Ringer. Let $O.D._2$ and τ_2 be respectively the optical density and turbidity of the fiber in a different solution. Consider first the case where $\tau_2/\tau_1 < 1.0$. (This corresponds to bathing the fiber in sucrose, tris, potassium or pH = 9.6 Ringer.)

From Eqn. [28] $O.D. = -\log_{10}(I/I_o)$ and from Eqn. [30]

$$I/I_o = \exp -(\beta + \tau)\ell$$

Therefore, $$O.D._2/O.D._1 = (\tau_2 + \beta)/(\tau_1 + \beta)$$

Now $\tau_2/\tau_1 < 1.0$

Hence $1 + \beta/\tau_1 < 1 + \beta/\tau_2$ or $\tau_2/\tau_1 < (\tau_2 + \beta)/(\tau_1 + \beta) = O.D._2/O.D._1$

Thus, the relative O.D. does not decrease to as low a value as the relative turbidity. Similarly, it may be shown that if $\tau_2/\tau_1 > 1.0$, the relative O.D. does not increase to as high a value as the relative turbidity.

APPENDIX II

Proof that the light scattered through small angles decreases the experimentally observed changes in optical density. Let the symbols have the same significance as in Appendix I and consider first the case where $O.D._1 > O.D._2$. If all other sources of error are ignored, $O.D._1 = c\tau_1(\ell/2.303)$ where $0 < c < 1.0$. Now, $O.D._2 > c\tau_2(\ell/2.303)$ for two reasons. There is less light scattered through small angles in the second bathing solution, and the relative importance of the scattered light is less because of the lower O.D. Therefore, $\tau_2/\tau_1 < O.D._2/O.D._1$. The relative O.D. does not decrease to as low a value as the relative turbidity.

Similarly, it may be shown that if $O.D._1 > O.D._2$, the relative O.D. does not increase to as high a value as the relative turbidity.