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THE HETEROGENEOUS DISTRIBUTION OF MONOVALENT CATIONS IN FROG SARTORIUS MUSCLES:

EVIDENCE FOR INTERNAL IONIC REDISTRIBUTION IN RESPONSE TO K-FREE RINGER TREATMENT AND TO OUABAIN TREATMENT

by

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ABSTRACT

Changes in intrafibre ionic activities and total intrafibre ionic contents were determined by ion-selective microelectrodes and by atomic absorption spectrophotometry respectively in the frog (Rana pipien) sartorius muscles which were exposed to solutions designed to perturb the normal distribution of monovalent cations. Free intrafibre K, fell 36mM after 40 minutes while total intrafibre K remained constant in muscles exposed to K-free Ringer. These muscles showed a 16mM increase in the free intrafibre Na but only a 5.5 m.moles/kg fibre water increase in the total intrafibre Na. Muscles treated with normal Ringer containing 10^{-4} M ouabain for 40 minutes showed a 27mM loss in the free intrafibre K and a 12 m.moles/kg fibre waterloss in the total intrafibre K. These ouabain treated muscles also displayed a 5mM increase in the free intrafibre Na and a 7m.moles/kg fibre water increase in the total intrafibre Na. Since the fall in free K did not equal the fall in total K, intrafibre K must be distributed heterogeneously.

The data were analyzed by means of a two compartment model for the distribution of monovalent cations. One of these compartments is defined as the myoplasmic space containing

the free cations; the other compartment becomes the remaining intrafibre space which contains the remaining cations which are either bound or at least inaccessible to the ion-selective microelectrodes. According to this analysis ouabain treatment produced losses of both free and bound K and increases of both free and bound Na; whereas exposure to K-free Ringer initiated an internal sequestration of free K and free Na by the "bound" compartment in exchange for an unknown ion.

Intrafibre pH, intrafibre Cl activity and total intrafibre Cl, total intrafibre Ca and Mg were also measured under K-free conditions in order to gain insight into the fundamental change which must have occurred to induce the internal K and Na shift from the free to the bound compartment. Although these experiments revealed little, they at least permitted one to suggest that K might be displacing Mg at a binding site.

The calculated fractional volume of the two compartments are 0.23 and 0.77 of the intrafibre water for the free and bound compartments respectively. On the basis of these volumes the second compartment was assigned to myofibrils and the free compartment to its surrounding myoplasm.

The results and analyses show that while ouabain and K-free Ringer both inhibit the normal operation of the Na pump as shown by the increase in the Na levels their effects on the overall electrolyte distribution were not identical.

The combined effects of ouabain and K-free Ringer were compared with that of K-free Ringer. Both experimental protocols gave equal falls of free K and similar increases of total intrafibre Na, however, the total K fell only when ouabain was present, indicating that ouabain and K-free Ringer operated by independent mechanisms. More importantly, the results of this thesis show that the myofibrils can act as a sink for monovalent cations such that during exposure to K-free Ringer the muscle fibres conserved its total intrafibre K at the expense of its free intrafibre K. But, during exposure to normal Ringer containing ouabain there was a 1:1 exchange of bound K for free Na.

Muscles that were previously exposed to K-free Ringer for 40 minutes when returned to normal Ringer for 40 minutes revealed that the expected extrusion of the accumulated Na did not occur. It was observed that the muscle fibres gained an additional 5m.moles/kg fibre water of total intrafibre Na while free Na remained constant. Total intrafibre K fell 5m.moles/kg fibre water while free K increased 21mM. Within the context of the two compartment model, returning a muscle to normal Ringer caused a release of the previously sequestered K into the myoplasm some of which then exits the muscle fibre by a 1:1 exchange for external Na. This influx of Na then enters the myofibrils. This unexpected redistribution of ions is discussed with respect to an altered intrafibre ionic distribution mediated by the initial exposure to K-free Ringer.

In a group of experiments designed to examine the mechanism of intrafibre sequestration of K during exposure to K-free Ringer it was observed that the sequestration was abolished by removal of external Na. That is, both free and total K remained constant in muscles exposed to K-and Na-free Ringer while both free and total Na fell. The intrafibre sequestration of K was also abolished by detubulating the muscles by the glycerol shock technique. These muscles did not exhibit significant changes in either K or Na levels when exposed to K-free Ringer. It was tentatively concluded that the internal sequestration of K may be initiated by an influx of Na across the tubular membrane.

In the last series of experiments, muscles exposed to Na-free Ringer showed decreases in both free and total intrafibre Na while both free and total K increased although not significantly. The fall in the Na levels was inhibited but not abolished by 10^{-4} M ouabain. Ouabain also initiated decreases in both free and total intrafibre K. This group of experiments shows that during exposure to Na-free Ringer part of the Na efflux exits the muscle fibre via the ouabain sensitive Na/K ATPase possibly linked to an influx of K. More importantly, the experiments of this thesis show that the frog skeletal muscle possesses a means of conserving its K when exposed to K-free Ringer but does not possess a corresponding means of conserving its Na in Na-free Ringer.

CONTENTS

FIGURES

TABLES

Chapter I

CONTROVERSIES IN THE STATE OF INTRACELLULAR WATER

1.1 PHYSICO-CHEMICAL PROPERTIES OF WATER

It will become apparent that in order to understand the findings of this thesis, that a brief review of the state of intracellular water and intracellular ions will be necessary. The state of intracellular water can be related to the unique physico-chemical properties of the water molecule. The water molecule consists of an oxygen atom and two hydrogen atoms. The two oxy-hydrogen bonds are 0.99 A long and are seperated by a 104.5° angle. This arrangement gives the water molecule its dipole moment [1] and its ability to form hydrogen bonds [2]. This hydrogen bonding effect is hypothesized to be a cooperative phenomenon, that is, the

[1] The dipole moment is a measure of the moment on a molecule for a given electric field. The dipole moment of water allows it to be oriented by an electric field such as one from a dissolved ion or a charged surface.

[2] The hydrogen bonding results from the marked differences in the electronegativity between oxygen and hydrogen atoms which causes an unequal charge distribution within the water molecule. Because of the oxygen atom's electronegativity or affinity for electrons, the electrons of the hydrogen atom tend to spend more time within the 2p orbitals of the oxygen atom. Thus completing the electron shell of the oxygen atom while the hydrogen atom is stripped to its bare proton. This sets up the assymetrical distribution of charge and allows the water molecules to form the so-called hydrogen bonds.

formation of one hydrogen bond promotes the formation of other hydrogen bonds. It is precisely the ability of water molecules to form hydrogen bonds and its dipole moment that gives rise to water's unique properties and structure. An in depth review of the properties and structure of water is beyond the scope of this thesis. The interested reader is referred to two excellent monographs (Eisenberg and Kauzman, 1969; and Tanford, 1980). However, a review sufficient for the reader to understand the anomalies of intracellular water is presented here.

Many models for the structure of water have been postulated, but none can account for all the properties of water. The most generally accepted model of water is that of Frank and Wen (1957). This model is based on the hypothesis that the formation of hydrogen bonds is a cooperative phenomenon. That is, as one hydrogen bond is formed others are encouraged thus forming clusters of water molecules. Conversely, as hydrogen bonds are broken by thermal motion neighbouring hydrogen bonds are destabilized. Thus one can envision a model of "flickering" clusters of water molecules. The cluster formation has been proposed to be ice-like (Nemethy and Scheraga, 1962). These authors have made quantitative calculations based on this model and have found that at 20 °C 70% of the water molecules lie in ice-like clusters with an average 57 molecules each. The structure as mentioned above is ephemeral with a half-life of 10^{-10} to 10^{-11} sec-

onds (Collie et al., 1948). Because of the transient nature of the water structure the viscosity of water is similar to that of non-associated liquids, however, physical properties such as boiling point and freezing point are much greater than those of non-associated liquids. It is intuitively obvious that factors which affect the structure of water will alter the physical properties of water.

The precise arrangement of liquid water is unknown, but the tetrahedral symmetry of the oxygen bonds [3] and the tetrahedral structure of ice suggests a local tetrahedral arrangement. This is supported by X-ray diffraction data (Tanford, 1980) which shows that liquid water has only 4.4 nearest neighbours. This is also supported by thermodynamic data (Tanford, 1980) where the heat of sublimation of ice is 11.2 kcal/mole at 0 °C, of this 9.8kcal/mole is the energy required to break the hydrogen bonds. However, the heat of fusion of ice is only 1.4kcal/mole. This is indicative that the hydrogen bonds in the solid state are maintained in the liquid state.

Pure water is isotropically distributed and the solution of any solute disrupts this distribution. For polar solutes, strong bonds between water and solute can form which may more than compensate for the disruption or distor-

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[3] A water molecule is capable of forming four hydrogen bonds. The two hydrogen atoms can each take part in forming a single hydrogen bond by being a hydrogen donor and the oxygen atom can act as an acceptor of two hydrogens. The tetrahedral structure is centered around the oxygen atom.

tion of the hydrogen bonds between water molecules. For this reason polar substances tend to be soluble in water. No such compensation occurs for nonpolar solutes hence their solution is thermodynamically unfavored accordingly. When a nonpolar substance is added to water, hydrogen bonds must first be broken to accomodate the nonpolar molecule then reformed around the nonpolar molecule with a different orientation. The net result is an increase in the ordering of water molecules in the vicinity of the nonpolar molecule. This explains the decrease in entropy observed when nonpolar solutes are dissolved in water (Frank and Evans, 1945).

Following this brief account, it can be readily appreciated that a complex macromolecule with both hydrophillic and hydrophobic regions must produce considerable breakage and reformation of the water structure before it can be accomodated in an aqueous environment. The end result is a new local order of water molecules close to the macromolecule with altered physical properties.

The muscle fibre contains many macromolecules, the most abundant group being those which form the contractile filaments, which may well alter the physical properties of a significant fraction of intrafibre water.

1.2 STATE OF INTRACELLULAR WATER

For most of this century, most biologists have been content to regard the cytoplasm as a simple homogeneous solu-

tion in which all cytoplasmic water acts as solvent for all cytoplasmic solutes. This simple model has admittedly explained many membrane phenomena very well. It has, for example, produced excellent theories for the resting membrane potential, the action potential, and active and passive transport. However, the fact that these theories are successful should not be used as evidence to support the initial assumption that the cytoplasm is indeed a simple homogeneous system because too much morphological and physico-chemical evidence has accumulated to indicate that such is not the case.

Evidence in support of the idea that cellular water may be non-ideal may be found as early as the turn of the century. Overton (1902), for example, immersed a muscle in a half normal osmotic strength solution and observed that the muscle did not swell to twice its initial size. He concluded that a substantial fraction of the fibre water was osmotically inactive. Later, Rubner (1922) measured a fraction of water which did not freeze at -20 °C and concluded that 23% of the fibre water was "bound" [4]. However, contrary to these observations, Hill (1939) found from his water vapour measurements that only 4% of the intrafibre water was bound in the frog muscle. But, as pointed out by McLaughlin and Hinke (1966), Hill had soaked a muscle in an equal volume of solution at two times the normal osmotic strength and deter-

[4] The term "bound" from hereon refers to water which deviates from ideal behaviour. It does not imply any thermodynamic state.

mined the vapor pressure of bathing solution. His determination of the vapour pressure was accurate but he did not consider the weight loss of 10-20% by the muscle. McLaughlin and Hinke (1966) by assuming a weight loss of 15% in Hill's muscles calculate that at least 27% of the total muscle water was bound. Unfortuately, because of Hill's conclusion the debate concerning the state of intracellular water was left dormant for years.

The abundance of complex cytoplasmic structure should make it obvious that there are many intracellular components which can affect the physical properties of intracellular water. For example, the surface of a membrane composed of lipids and proteins presents a charged surface with a charge density of approximately 1 electron charge per 300 A^2 (McLaughlin, 1977). Clearly, such a surface will alter the orientation and the physical properties of water molecules in its vicinity. It is important to study the properties of intracellular water because : (i) in order to properly define transport rates one must know the appropriate fraction of water involved; (ii) the stability of the myofilaments may depend on the properties of water (Morod and Gingold, 1979); and (iii) the activity of enzymes are related to their hydration (Yang and Rupley, 1979; and Bone and Pethig, (1982). The following will be a summary of data which demonstrates that part of the intracellular water must be bound.

The exact degree to which intracellular water is bound is currently unresolved and dependent on the experimental method employed. For example, the degree of hydration[5] of a variety of crystallized proteins as measured by diffusion and viscosity has been summarized by Tanford (1961, p.359) and Tanford(1961, p.395) respectively. The values given in these tables contain the caveat that they are the maximum values calculated by assuming that the proteins are spherical, nevertheless, even a cursory examination of the values produced by these two techniques reveals a large discrepancy in the amount of bound water for every protein listed. Both these techniques do show a significant fraction of bound water. For example, the water of solvation of hemoglobin measured by diffusion was 0.36g H_2O/g protein; whereas that measured by viscosity was 0.69g H_2O/g protein. Savitz et al. (1964) made volume measurements on red blood cells and found that these cells do not behave as a perfect osmometer. This deviation from ideal behavior could be accounted for if 20% of the cellular water was assumed to be osmotically inactive. These authors calculate that 12g H_2O/100ml of cell participates as the water of hydration for hemoglobin (based on a hemoglobin content of 35g/100ml cell and by assuming a water of solvation of 0.3gH_2O/ghemoglobin). Since the water content of their cells were 72g/100ml cell

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[5] No distinction is made here between bound water and the water of hydration. The discussion is merely meant to illustrate that the measured amount of water affected by the protein is dependent on methodology.

it appears that the water of hydration is in fact also the fraction of water which is osmotically inactive.

Volumetric measurements on single fibres from the frog semitendinosus have shown that 35% of the fibre volume is osmotically inactive (Reuben et al., 1963). Blinks (1965) also made volumetric measurements on isolated fibres of frog tibialis anterior muscles and found that 33% of the cellular volume was osmotically inactive. In addition, Blinks (1965) calculated that 20% of the intrafibre volume is occupied by solids. Rome (1968) measured the myofilament lattice volume of toad muscles by X-ray diffraction and found that approximately 38% of the volume of the sarcomere was osmotically inactive. Thus it appears that almost 20% of the intracellular water of amphibian skeletal muscle is osmotically inactive and that this osmotically inactive water is located in the myofibrils. Hinke (1970) measured the osmotic behavior of single barnacle muscle fibres by two techniques and found that 27% and 28% of the total intracellular water was osmotically inactive. He also measured the solvent water for free Na, K and Cl by five techniques and reported that 22 to 29% of the total water was not available as solvent. Caille and Hinke (1974) measured the volume available for the diffusion of water in the barnacle muscle fibre and found that 20% of the fibre water was not available for diffusion. Evidently, a significant fraction of water in a muscle cell deviates from ideal behavior. Furthermore, it

appears that the solvent water for free ions, the osmotically active water and the water available for diffusion may be identical.

Nuclear magnetic resonance (NMR) has also been used extensively to study the properties of intracellular water. There are two principle models used to explain the NMR data. In one model, intracellular water is essentially identical to pure water with only a small fraction bound by intracellular constituents. In this model the observed deviation of the NMR parameters of intracellular water from that of pure water is caused by a fast exchange between the small bound fraction and the bulk fraction. In the second model, a large fraction of the intrafibre water is bound and accordingly the physical properties of a significant fraction of water is altered. There is a vast amount of data from the proponents of both models.

Proponents of the first model state emphatically that only a small fraction of the intracellular water is actually bound. For example, Cooke and Wein (1973) report data to support this view using glycerinated rabbit psoas muscles dried to varying degrees of hydration. They reasoned that if the exchange of water between intracellular phases is slow compared with the decay of magnetization of water protons then the decay should follow a sum of exponentials. However, if the converse were true then the decay should follow a single exponential. Furthermore, these authors

argue that the rate of decay of magnetization should decrease with the decrease in the degree of hydration - thus indicating that the bound water is responsible for the fast decay of the water protons invariably observed by NMR [1]. These authors found that the decay of the proton magnetization followed a single exponential and also that the relaxation times decreased as the degree of hydration decreased. They conclude from these observations that the behavior of intracellular water agrees with the classical theory of cellular water, that is, the cell is essentially a salt solution with a very small fraction of bound water (4-5%). These workers also cite the small decrease in the intracellular diffusion co-efficient of water as support for a fast exchange model. Finch and Homer (1974) also used a two compartment fast exchange model to analyze their data on the frog gastrocnemius muscle. These authors calculate that 97% of the intracellular water has identical motional freedom as that of ordinary water and only 3% of the water had its motion restricted to between that of liquid water and ice. Foster et al. (1976) working on barnacle muscle fibres observed that only 1 water molecule per 1,000 (0.1%) is so tightly bound as to lose its translational and rotational motion. These bound water molecules have a Brownian motion

[1] The consistent observations made for water protons in various tissues in comparison to pure water are: (i) the spin-lattice relaxation time, T1, does not equal the spin-spin relaxation time, T2; (ii) T1 is reduced by a factor of four or five ; (iii) T2 is reduced by a factor of about 50; and (iv) the self diffusion co-efficient of water is reduced by a factor of 2.

essentially identical to that of the protein to which it is bound. The bound time of these water molecules are tens of microseconds, consistent with a fast exchange model.

The small fraction of bound water is in apparent agreement with that found in protein solutions. Kuntz et al. (1969) working on bovine serum albumin, ovalbumin, lysozyme and α-chromotrypsin found that 0.3-0.5g H_2O/g protein was bound. This corresponds to approximately 7% of intracellularly bound water. The data of Foster et al. (1976) is also in agreement with the data of Koenig and Schillinger (1969 a and b) and Fabry et al. (1970). That is, only a very small number of water molecules are so tightly bound as to have the same Brownian motion as that of the protein. However, as stated by Koenig in response to Cooke and Wein (1973) the water of the first hydration sphere consists of 10-20 molecules that bind sufficiently well to make their Brownian motion essentially identical to that of the protein. These water molecules do, however, exchange rapidly with the surrounding water with a time constant between 0.1-10usec. However, there is a layer of water around the protein with a tumbling time one-hundred times slower than that pure water but one-hundred times faster than that of the protein. This fraction of water was 10-30% of the weight of the protein. It is in this fraction that the controversies lie. There is no doubt that only a small fraction of the intracellular water is tightly bound such that its translational motion is

identical to that of protein. As concluded by Swift and Barr (1973), all of the muscle water is NMR visible but only a small fraction is tightly bound but a significantly larger fraction of the muscle water has some motional restriction. It is apparent that different experimental paradigms measure different fractions of bound water because they are measuring different degrees of binding. Perhaps the relevant question to this thesis is what are the fractions of free and bound water which participates in the non-uniform distribution of intracellular monovalent cations.

Proponents of the second model state that a significant fraction of water is affected by the protein in closer agreement with the osmotic experiments than proponents of the first model. Hazlewood et al. (1969) found that the NMR signal of rat skeletal muscle was consistent with a significant fraction of structured intracellular water. The observed structuring could be removed by denaturing the muscle proteins through heating. These investigators concluded that the intracellular structuring of water was dependent on the native conformation of the muscle proteins. Belton et al. (1972) measured the NMR transverse relaxation times in frog gastrocnemius and sartorius as a function of temperature. Three fractions of water were observed: (i) the fraction with the least motional restriction had a fractional size of 15% was identified as water of the extracellular space; (ii) the fraction with intermediate motional restric-

tion had a fractional size of 65% and was identified as the bulk intracellular water; and (iii) the fraction with the greatest motional restriction had a fractional size of 20% was identified as the water "strongly" bound to proteins. This bound fraction did not freeze at temperatures as low as -30 °C and is in good agreement with the fraction of the non-osmotic water discussed above. There was a loss of approximately 80% in the NMR signal at about -8 °C indicative that at -8 °C freezing of the bulk of the intracellular water had occurred and that the remaining 20% was prevented from undergoing the liquid/solid transistion. The mechanism by which water molecules are prevented from joining the ice lattice will be discussed below.

In an attempt to study the molecular organization of bound water Fung (1975) and Kasturi et al. (1980) studied the anisotropy of their NMR spectra. Fung (1975) discovered that the spectra of the frog gastrocnemius exhibited an angular dependence and concluded that there must be a favored orientation for muscle fibre water. Kasturi et al. (1980) studied the temperature dependence of the anisotrpy and observed a decrease in anisotropy with temperature. They discovered that as water froze the remaining unfrozen water (25% at-5 °C and 20% at -10 °C), showed a decreasing anisotropy from which they concluded that the anisotropy resided more in the frozen water. In other words, the preferred orientation of water was a long range effect of the contrac-

tile proteins. Peemoeller et al. (1980) also conducted temperature studies on mouse muscles and found the non-freezing water to be in a semi-liquid state at room temperature which undergoes a phase transition to the solid phase at -27 °C into a polymer of water different from ice. This is additional evidence that a fraction of the cell water is affected by cellular constituents such that its structure is different from that of ideal water.

Self-diffusion co-efficient measurements by NMR (Chang et al., 1973) have shown the intracellular diffusion of water to be an activated jump process similar to that of pure water and ice. The process involves the breaking of two hydrogen bonds in intracellular water and in pure water and indicates a semi-crystalline structure. The energy of intracellular water hydrogen bonds was essentially identical to that of pure water. The reduction of diffusion co-efficient could not be accounted for by the compartmentalization of water by the sarcolemma or by the sarcoplasmic reticulum (Chang et al., 1973) nor by obstruction by the myofilament lattice (Rorschach et al., 1973; and G.C.Cleveland et al., 1976). It was concluded by Chang et al. (1973) that the reduction in the self-diffusion co-efficient was due to a change in structure of the water molecules by the presence of macromolecules. This conclusion is supported by Finch and Homer (1974) who observed that a portion of the intracellular water had a decreased entropy

and by Harvey and Hoekstra (1972) who also observed a decreased entropy in the water of hydration of lysozymes. This is indicative of an increased order or a preferred orientation of intracellular water molecules. Thus macromolecules alter the properties of its vicinal water not by changing the bonding characteristics of the water molecules but by increasing the local order of the water molecules. It should be apparent from the preceeding section that these are the properties of the hydrophobic effect and that the alteration of the struture of water may alter its physical properties without any demonstrable changes in some of its motional freedoms. Thus there could exist a fraction of water which could be bound such that its physical properties are altered yet this fraction could remain undetected by techniques employed by proponents of the first model of intracellular water. This point will be elaborated on below.

The current controversies in the state of intracellular water has been summarized by comprehensive experiments on single barnacle muscle fibres by Burnell et al. (1981) and by Clarke et al. (1982). These workers made NMR measurements and found that the magnetization relaxation experiments (Burnell et al., 1981) gave values of hydration an order of magnitude lower than that measured by self-diffusion experiments (Clarke et al., 1982). As pointed out by Clarke et al. (1982) the calculated value of hydration is

substantially affected by the model chosen. In addition, they show that the measured degree of hydration is dependent on experimental technique employed. These authors pointed out the three aspects of hydration that are measured: (i) thermodynamic aspects which relate to enthalpies and entropies of molecular interactions ; (ii) dynamic aspects which correlate with molecular motions; and (iii) structural aspects which are correlated with the geometric arrangement of solvent molecules. This was illustrated very well by Pezolet et al. (1978) who used Laser Ramam Spectroscopy to compare the molecular structure of intracellular water with that of pure water. They observed that no more than 5% of the intracellular water of the barnacle muscle fibre could have structure and hydrogen bond energy different from those of of pure water, yet they also observed that at -10 °C 20% of the water remained unfrozen.

NMR experiments measure the motional aspects of water, that is, the translational and the rotational mobility of water molecules and these properties are not related to solvent, osmotic or freezing properties which are thermodynamic aspects. The NMR technique employed by Burnell et al. (1981) measures only that fraction of water with greatly reduced translational and rotational mobility (i.e. tightly bound water). Accordingly, they measure only a small fraction of bound water (0.07g H_2O/g macromolecule). In contrast, the self-diffusion co-efficient determinations of

Clarke et al. (1982) measures that fraction of water with less severely restricted mobilities (i.e. loosely bound water). Accordingly, they measure a larger fraction of bound water (0.65g H_2O/g macromolecules). Clarke et al. (1982) proposed that the molecular interaction between water and macromolecules results in a gradient of water mobility with virtual zero at the surface of the macromolecules and increasing with distance away from the macromolecules. These authors also proposed that the reduced self-diffusion of water is mediated by long range hydrophobic effects ofthe non-polar groups on the macromolecule which produces the extensive ordering of intracellular water. Furthermore, it is this ordering that reduces the translational motion of the water molecules that is manifested in the reduced self-diffusion of water.

Recently, water tightly bound to crystallized lysozymes have been studied with X-ray analysis (Blake et al., 1983). They carried out their analyses on crystals of human lysozyme and tortoise egg white lsozyme and found that 140 water molecules and 128 water molecules were bound respectively as to lose their translational motion (X-ray analysis provides structural information on molecules that have lost their translational motion it is insensitive to rotational motion). This level of bound water corresponds to a hydration of about 0.1g H_2O/g protein. This is similar to the value of 0.2g H_2O/g protein (Burnell et al., 1981). These water molecules were bound to the polar groups of the pro-

tein molecules by hydrogen bonds; 33-35 of the water molecules formed two or more hydrogen bonds to the proteins and the remainder formed one hydrogen bond to either the protein or the double bonded water. These numbers are in good agreement with those observed by Bone and Pethig (1982) who used dielectric relaxation measurements on hen egg white lysozyme and found that 32 water molecules were so tightly bound to the lysozyme (with two hydrogen bonds) as to lose their rotational and translational. In addition, there were 69 water molecules with one hydrogen bond to either the lysozyme directly or to the double bonded water. This latter fraction of the water loses its translational motion but retains its rotational motion.

Structural information from X-ray analysis (Blake et al., 1983) indicates that the tightly bound water exists in two forms. The primary form has hydroben bond angles between 100 and 110 degrees, indicating a tetrahedral packing. The secondary form has hydrogen bond angles between 70 and 80 degrees, indicating a triangular structure. These workers argue that since the water structuring was similar in four types of lysozymes with different crystalline structure and that the water structure was independent of the salt present, the water structure, therefore, must be a function of the lysozyme. Furthermore, they argue that this bound water is reflective of the structure of bound water in a solution of macromolecules. If this hypothesis is correct

then one has a structural model for the fraction of tightly bound water.

It is clear that the state of intracellular water is controversial and unresolved. There is currently no theory which links the various aspects of water (i.e. the thermodynamic, the structural and the dynamic aspects). The NMR data suggest that only a very small fraction (about 4%) of the intracellular water is so tightly bound to the macromolecules as to lose all of its Brownian motion. This fraction, however, exchanges rapidly with the remaining intracellular water. X-ray analysis suggests that this water is hydrogen bonded to the polar groups of the protein or hydrogen bonded to these bound water molecules. In addition, this water appears exists in two forms, a primary tetrahedral structure and a secondary triangular structure. There is, in addition, to this fraction of tighly bound water a fraction of intracellular water that is loosely bound and retains some of its motional freedom. It was suggested by Clarke et al. (1982) that there exists a gradient of motional freedom with zero motional freedom for water molecules at the surface of the protein to total motional freedom for those in the bulk solution. It follows that there must also be a gradient of binding and hence there must also be a gradient in the alterations to the properties of the intracellar water.

Pezolet et al. (1978) have shown that 20% of the intracellular water does not freeze at a temperature significantly below that of pure water. This resistance to freezing occurs without a large alteration in the structure or the hydrogen bonding properties of water. This indicates that the alteration to the intracellular water must be subtle yet it prevents the liquid/solid phase transition. Poemoeller et al. (1980) have shown that this unfrozen water is in a semicrystalline state different from the structure of pure water. This alteration was due to a decrease in the local entrophy of water molecules in the vicinity of the proteins (Harvey and Hoekstra, 1972). Clark et al. (1982) suggested that this is mediated by the hydrophic portions of the proteins.

Alteration to the structure of water will affect its physical properties. For example, a significant fraction of the intracellular water have been shown not to participate in the solvation of ions nor to be osmotically active. Yet, there is evidence that bound [7] water can act as solvent water (Vargi-Manya, 1979). Thus it is not clear how solvation water is related to ideal water measured by various methods. Therefore, it would not be productive to argue what the exact fraction of "bound" water is rather one should be concerned with the appropriate fraction of water that deviates from that of pure water with respect to the

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[7] Bound water here refers to water that migrates with a charged molecule in an electric field.

pararameter under study.

For this thesis, it is enough just to know that the solvent water for the free ions in the muscle (as measured by the ion-selective microelectrode) need only be a fraction of the total analyzable intrafibre water. It will emerge from the analysis of the experimental data that the remaining fibre water (that not related to the free ions) is substantial but it will not be necessary to decide on its exact physico-chemical state.

Chapter II

HETEROGENEOUS DISTRIBUTION OF INTRAFIBRE IONS

2.1 BINDING OF IONS BY CONTRACTILE PROTEINS AND BY MEMBRANELESS MUSCLE FIBRES

It has been established that the structure and properties of intrafibre water can be affected by the macromolecular structure of the cytoplasm. One of the properties that may be altered as water increases its structural order is its ability to solvate ions. In addition, many of the cytoplasmic macromolecules may have an profound effect on the mobility of ions because of their charge at physiological pH. Biological polyelectrolytes particularly the contractile proteins and DNA bind ions *. The binding of ions by DNA in frog skeletal muscles is insignificant compared to that of the contractile proteins and will not be discussed in this thesis. The interested reader should consult the following references : (i) Wilson et al. (1980); (ii) Ander-

* A rigorous treatment of the mechanisms of ion binding will not be explored in this thesis. Suffice it to say that the "association" of ions to polyelectrolytes can be divided into two categories: (i) ions that are site bound (i.e. ions that have lost their water of hydration) and are restricted to the vicinity of a charged group; (ii) ions that are loosely or territorially bound by the electrostatic field of the polyelectrolyte and have retained most of their water of hydration. This latter group retains most of its freedom and mobility within a domain around the polyelectrolyte.

son et al. (1978); and Schellman and Stigor (1977).

Ling's Association-Induction Hypothesis (1962) and Troshin's Sorption Theory (1960a) have stimulated a number of studies to prove that the entire cytoplasm is a highly ordered and semi-crystalline state and that such structure is totally responsible for the selective and unique accumulation of ions and solutes. The principle preoccupation of Ling and his disciples in the last quarter century has been to prove that the K ion is selectively bound to the ordered cytoplasm. Their primary intention has been to discredit the Membrane Theory rather than to look objectively at the problem of ion binding, detached from all theories and/or hypotheses.

In order to avoid a fruitless discussion on the existence or non-existence of the plasma membrane and its pumps, it was decided to neglect many interesting experiments carried out by Ling and his disciples. It should at least be mentioned here, however, that this group of investigators were instrumental over the past twenty-five years in keeping alive the important question of water structuring and ion binding during a period in biology when most cell physiologist and biochemist preferred not to consider such complexities.

It is known that extracted contractile proteins (Szent-Gyorgi, 1948) and muscle homogenates (Steinbach, 1950) bind ions. However, Lewis and Saroff (1957) were the first to

examine the monovalent cation binding properties of these proteins in a rigorous manner. They have shown that the monovalent cations are bound in a pH dependent manner. They calculated a maximum binding capacity of about 50 eq/10^5g myosin which is equivalent to 60 m.moles/kg muscle wet weight. Interestingly, Lewis and Saroff also correlated the loss of ion binding with a change in sedimentation as the temperature was increased. This suggests that the binding of monovalent cations was dependent on the tertiary and quaternary structure of the contractile protein. The binding of monovalent cations by contractile protein has also been observed by Edelman (1980 and 1981) in freeze dried muscles using both X-ray and laser microanalysis. It can be argued that extracted proteins do not represent a good model for the structure of proteins in a cell especially the contractile proteins with its cross-linked matrix and ,therefore, cannot reflect the true state of ion binding in a muscle fibre. It nevertheless demonstrates that contractile proteins can bind monovalent cations hence may cause the non-uniform distribution of intrafibre cations.

Muscle fibres with destroyed membranes have also been used to study the charge density of the contractile apparatus and its effects on ions. The methods of membrane removal include glycerination, mechanical removal and detergent treatment. Rome (1968) in a study of the lattice dimension of the myofibrils in glycerinated muscles found that the

lattice dimension was a function of pH, ionic strength and the composition of the medium. This dependence of lattice volume on the electrical parameters of the bathing medium suggests the presence of electrostatic forces on the contractile proteins. Furthermore, it was suggested from the pH dependence and the ionic composition dependence that the electrostatic forces arose from the presence of ionizable groups on the contractile proteins. It follows that the existence of an electrostatic potential on the contractile proteins must influence the behavior and distribution of ions in solution including a solution such as the myoplasm.

Collins and Edwards (1971) analyzed the electrostatic potential and the charge density at the surface of the myofilaments in glycerinated muscles by measuring the Donnan potential using microelectrodes filled with KCl. They found that the measured potential varied with pH and ionic strength. The variations with ionic strength was as expected for a Donnan potential. This potential could be decreased by partial extraction of the contractile proteins in 0.6 M KCl and could be made to reverse sign when the pH

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* The dimensions of the tips of the microelectrodes used in such studies are typically 0.1 um (i.e. 100nm); whereas the dimension of the filaments are about 10nm and the distances seperating the filaments are about 40nm. It is obvious that the microelectrodes will actually measure only an averaged electrostatic potential within the myofibrils and not the true Donnan potential between two solutions seperated by a sharp boundary. However, Elliott and Bartels (1981) and Naylor (1981) have shown that under physiological conditions the analysis of charge by the Donnan approach is identical to the analysis of charge by the electostatic approach.

was lowered from 7.5 to 4.0 (the isoelectric points of the contractile proteins are about 5). Furthermore, these authors measured the activity of K in the myofibrils and in the surrounding bath and found them to be in thermodynamic equilibrium. These observations led these authors to conclude that the Donnan potential was caused by the contractile proteins. The important implication of a Donnan potential is that one or more mobile ionic species is restricted in its mobility and this leads to regional differences in the concentration of mobile ions. This conclusion is corroborated by the work of Fenn (1957) who by chemical analysis demonstrated an accumulation of Na by glycerinated fibres. Many other investigators have also measured the Donnan potential in membraneless muscle fibres (Weiss et al., 1967; Pemrick and Edwards, 1974; Elliott, 1973, and 1980; Elliott et al., 1978; Caille, 1981; Bartels and Elliott, 1980 and 1981; Stephenson et al., 1981; and Hinke, 1980) and have also found a similar dependence of the Donnan potential on pH and ionic strength. Hinke (1980) and Stephenson et al. (1981) have also combined the potential measurements with chemical analysis. Hinke (1980) found that at physiological pH there was a negative Donnan potential between the myofibrillar phase and the surrounding fluid which correlated with his observation by chemical analysis that the myofibrils accumulated cations and excluded anions. From his pH dependence studies he deduced that the isoelectric point

from the potential measurements corresponded with the isoelectric point of the contractile proteins. Furthermore, it was also shown that as the measured potential reversed sign, anion accumulation was observed rather that anion exclusion. Hinke (1980) also made ionic activity measurements of the myofibrillar phase and confirmed the observation of Collins and Edwards (1971) that the myofibrils are indeed in equilibrium with the surrounding fluid and that the non-uniform distribution of ions must be a non-energy requring process. Stephenson et al. (1981) extended the chemical analysis to a large variety of ions and neutral molecules and demonstrated that the accumulation of cations and exclusion of anions at physiological pH were phenomena not restricted to inorganic ions but a general characteristic of the Donnan equilibrium. Indeed such behaviour is expected from polar molecules since Elliott (1978) reported that the potential gradient at the surface of the myofilaments is in the order of 10^7 V/m arising from a charge density of about 1e/50 A and 1e/150 A for the thick and thin filaments respectively.

It is clear from the foregoing discussion that both the extracted contractile proteins and membraneless muscle fibres can indeed bind significant quantities of ions and it follows that the myofilaments in an intact muscle must also bind ions and hence cause a non-uniform distribution of ions. Indeed the intrafibre binding of ions may be substantial since the myofibrils occupy about 83% of the intrafibre

volume of the frog sartorius muscle (Mobley and Eisenberg, 1975).

2.2 HETEROGENEOUS DISTRIBUTION OF MONOVALENT CATIONS AND ITS INTRACELLULAR LOCATION

The first indication that monovalent cations might not be homogeneously distributed in an intact muscle fibre came from experiments of Tobias (1950) who found that a fraction of the Na could not be leached out by prolonged soaking in distilled water. This was corroborated by tracer kinetic studies of Harris (1952 and 1953) who reported that a portion of the K from a frog skeletal did not exchange with externally applied tracers and by tracer kinetic studies of Conway and Carey (1955) who observed that a portion of the Na in a frog skeletal muscle did not exchange with externally applied tracers. Harris and Steinbach (1956) extended the experiments of Tobias and concluded that a fraction of the monovalent cations existed in an adsorbed state. Troshin (1957) and Sorokina (1964) calculated from their tracer studies that about 30% of the intrafibre Na was not free. Hinke (1970) also used tracer studies and calculated that perhaps as much as 44% of the total intrafibre Na in an intact single barnacle muscle fibre may either be bound or compartmentalized.

Two hypotheses have been put forth for the intracellular location of the bound monovalent cations. In one hypothe-

sis, membrane limited organelles, in particular the sarcoplasmic reticulum are responsible for the comparmentalization of ions. This hypothesis can now be laid to rest. Somlyo et al. (1977a, b and 1981) have shown by X-ray microprobe analysis that the sarcoplamic reticulum, the mitochondria and the nuclei do not concentrate monovalent cations above the levels of the cytoplasm. It should be noted that these workers did not provide a precise definition of their cytoplasm, that is, were the myofibils included. However, this caveat will not alter the conclusion that the membrane limited organelles are not responsible for the heterogeneous distribution of monvalent cations.

The compartmentalization of Na as suggested by the tracer studies have also been corroborated by experiments combining the Na-selective microelectrode with whole muscle chemical analysis. Hinke (1959) performed the first of such experiments on crab and lobster muscle fibres and found that the concentration of Na measured by chemical analysis exceeded that found by the Na-selective microelectode using any reasonable value for the intrafibre activity co-

[10] Ionic concentrations were calculated from the activity measurements by the following relation : $(aNa) = \gamma_{\pm}(cNa)$ where (aNa) is the activity of Na, $\gamma_{\pm}$ is the activity co-efficient and (cNa) is the concentration of Na. $\gamma_{\pm}$ can be calculated from the Debye-Huckel theory which relates the activity co-efficient to the concentration. Robinson and Stokes (1955) should be consulted for a treatment on this subject. It is reasonable to assume that the binding of ions by macromolecules will not affect the activity co-efficient of the free ions (Lewis and Saroff, 1957). Thus Hinke (1959) was justified in using the activity co-efficient of an electolyte solution which approximates the composition of the myoplasm for

efficient [10]. However, these experiments were only considered to be preliminary by Hinke since the number of measurements were too small. Lev (1964) and McLaughlin and Hinke (1966) also carried out similar experiments on frog skeletal muscle fibres and single barnacle muscle fibres respectively. Both confirmed that chemical analysis gave values of Na concentration much higher than the Na concentration measured by the microelectrode. Allen and Hinke (1970) combined the microelectrode measurements and chemical analysis with ^{22}Na isotope efflux and concluded that between 40 and 50% of the total intrafibre Na was free. These experiments provide good evidence that a significant fraction of the intrafibre Na is not in a free state.

The distribution of intrafibre K is more uncertain than that of intrafibre Na. It was stated above that Harris (1952 and 1953) suggested that part of the muscle K existed in a bound state. This postulate has been supported, for example, by the high frequency conductance measurements of Masszi and Tigyi-Sebes (1962) who found a significant portion of the intrafibre K that behaved as if it were bound. However, combined measurements of the K-selective microelectrode and of total muscle chemical analysis have shown that the free K concentration and the total intrafibre K concentration were very similar (Lev, 1964; McLaughlin and Hinke, 1966; Hinke and Gayton, 1971, and Armstrong and Lee, 1971). Thus it is debatable whether intracellular K is bound. How-

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the activity co-efficient of the free intrafibre ions.

ever, as pointed out by Hinke and McLaughlin (1966) and later by Lee and Armstrong (1974) the fraction of the free ion determined by microelectrodes is subject to variation depending on the fraction that one chooses as solvent water [11]. Armstrong and Lee (1971) and Lee and Armstrong (1974) provided the first strong evidence that intrafibre K is not distributed homogeneously. They reported changes in the free and total K and Na during prolonged exposures to K-free Ringer that were incongruous with a homogeneous distribution of K where K existed entirely in the free state. Briefly, these investigators found that changes in total K and Na were much greater than the changes in free K and Na. It follows that changes in the K and Na levels must have involved at least two compartments. Furthermore, the magnitude of these changes were consistent with a 1:1 exchange of bound K for Na. This observation led these investigators to conclude that there must exist a fraction of bound K in the normal frog skeletal muscle.

The location of the bound monovalent cations as suggested by the discussion at the beginning of this chapter are the myofibrils. It has been shown that extracted contractile proteins and membraneless muscle fibres can sequester

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[11] The fraction of water chosen as solvent water will not alter the conclusion that intrafibre Na is bound. It will only change the exact numerical value which is inversely proportional to the fraction of solvent water. It should be apparent from the discussion of the preceeding chapter that an exact value for the fraction of intracellular water with ideal solvent properties cannot be provided.

monovalent cations. In contrast, membrane limited organelles have been shown not to sequester monovalent ions. Hinke and McLaughlin (1967) measured the Na and K levels in a barnacle muscle fibre as the temperature was increased and found that the muscle fibres shortened irreversibly between 37-40 °C. Concomitant with this shortening was a loss of total intrafibre K and Na, while the Na activity increased. In addition, intrafibre pH fell while free intrafibre K remained constant. It should be noted that the increase in Na activity occurred while the muscle fibres were in Na-free Ringer. From this observation, they concluded that the increase of free Na could only have come from an intrafibre source. Since this increase in free Na coincided with the irreversible shortening of the muscle fibre, these authors concluded that the contractile proteins must be the source of the Na and H ions. The fact that the contractile proteins do release their bound ions during thermal denaturation was first reported by Szent-Gyorgi (1951). It should also be remembered that Lewis and Saroff (1957) correlated the loss of monovalent cation binding by the contractile proteins with a change in the sedimentation value as the temperature was increased. Finally, Aronson (1966) observed that at a critical temperature glycerinated muscle fibres undergo a loss of A-band birefringence which was correlated with the loss of A-band structure observed by electron microscopy. Thus McLaughlin and Hinke (1967) were justified

in concluding that the increases of Na and H ions during their temperature dependent shortening were indeed due myofibrillar denaturation. It should be pointed out that Hinke and McLaughlin (1967) were preoccupied with locating the bound Na and did not examine the possibility of myofibrillar K binding. The fact that they observed a significant fall in total intrafibre K while the K activity remained constant during thermal denaturation is evidence for myofibrillar bound K. The compartmentalization of Na by the myofibrils is supported by optical density measurements of McLaughlin and Hinke (1968). The optical density of a polyelectrolyte is related to the degree of ion binding. These workers observed a loss of optical density when muscle fibres were exposed to Na-free Ringer. This result is consistent with a loss of myofibrillar bound Na.

It is clear that the myofilaments can influence the behavior of ions and water. Caillé and Hinke (1972, 1973, and 1974) examined the diffusional profiles of Na, K, Cl, sorbitol and water in single barnacle muscle fibres. They compared these diffusional profiles with the theoretical profiles for a simple system under identical geometric conditions. From these analyses, Caille and Hinke concluded that a significant fraction of water did not diffuse and that a significant fraction of Na and K were condensed or bound to polyelectrolytes. In support of ion binding rather than some form of active sequestration by a membrane limited organelle as the cause of the deviation was the temperature

dependence of this deviation which was compatible with a non-energy requiring diffusion of an electrolyte. These investigators also examined the pH dependence of the diffusional profiles and found that at pH 5.2 (the isoelectric point of the contractile proteins) the experimental and theoretical profiles were identical. The deviation between the theoretical and experimental profiles resembles the pH dependence of the Donnah potential in membraneless muscle fibres. Notice also that the pH at which diffusion of monovalent cations is unimpeded also coincides with the isoelectric pH of the contractile proteins. From this disscussion it seems safe to conclude that the non-uniform distribution of monovalent cations in a muscle fibre is caused by the ion binding properties of the myofilaments.

Ion binding and ionic heterogeneity is by no means a special or unique property of the muscle fibre because of the presence of the myofibrils. For example, Hinke (1959) combined ion-selective microelectrodes with total chemical analysis and found that 24% of the Na and 10% of the K in a squid axon is probably bound. This binding of ions was also demonstrated in the extruded axoplasm and therefore cannot be a property of the membrane. The squid axon is essentially devoid of organelles and presumably the heterogeneous distribution of ions is effected by the macromolecules of the cytoskeleton. The non-uniform distribution of monovalent cations ions has also been demonstrated convincingly in

the salamander oocyte by Horowitz et al. (1979) and by Horowitz and Paine (1979). These workers also concluded that the non-uniform distribution of ions was caused by adsorption by the macromolecules presumably in the yolk platelets. If one now accepts the premise that significant quantities of monovalent cations are bound to the contractile proteins in the muscle fibre, how will the ion binding affect our thinking about membrane phenomena such as the Na pump. This will be examined in the following chapter.

Chapter III

THE NA/K PUMP AND HETEROGENEITY

The last two chapters have established that part of the intracellular water and part of the intracellular monovalent cations are bound by the cytoplasmic proteins. This chapter will explore the effect that such binding may have on our thinking of the Na/K pump. It is well established that the Na/K pump transports Na and K across the plasma membrane. Intuitively speaking when the Na/K pump moves Na and K across the plasma membrane it must also have an effect on all fractions of intracelluar Na and K or conversely speaking the heterogeneous distribution of monovalent cations must also affect the function of the Na/K pump.

The sodium pump was originally proposed as a mechanism to explain the cell's maintenance of an internal composition low in Na and high in K in an environment high in Na and low in K when it was learned that both K and Na could indeed permeate the plasma membrane (Dean, 1941). In intact tissues, for example, it was observed that Na entered cells in exchange for intracelluar K when extracellular K was low (Heppel, 1939 and 1940) or following activity or fatigue (Fenn, 1938). Steinbach, (1940) found that sartorius muscles from Rana pipiens lost K in exchange for Na when stored

in K-free Ringer. He also observed that this (K)i-(Na)o exchange could be reversed when the muscles were returned to K containing Ringer (10mM). Desmedt (1953) observed a similar (K)i-(Na)o exchange when he soaked the sartorius muscle for 68 hours in 0.2 mM K Ringer rather than K-free Ringer,[12] and a similar reversal to normal when he returned the muscle to K containing Ringer. Thus from these observations, it was concluded that the muscle fibre must have a mechanism to preferentially accumulate K and to extrude Na. The sodium pump has been intensely studied and a complete review of all of its properties is beyond this thesis. Since the primary objective of this thesis is to examine the effects of K-free Ringer and ouabain on the internal distribution of monovalent cations this chapter will focus on literature which describe the effects of K-free Ringer and ouabain on the Na/K pump and how compartmentalization of Na and K may affect our understanding of the Na/K pump.

The K concentration dependence of the Na/K pump was first studied carefully in the erythrocyte by Glynn (1956) who observed a linked Na and K transport which was dependent on the external K concentration. He observed that the uptake of K and extrusion of Na were completely abolished by K-free solutions. Furthermore, Glynn observed that glucose removal abolished this linked Na and K transport to the same degree as K-free Ringer. Thus it appears that K removal has

[12] Desmedt used 0.2 mM K rather than K-free Ringer because K-free Ringer was observed to produce irreversible alterations in membrane characteristics.

abolished the active uptake of K and extrusion of Na. The K concentration dependence of the Na/K pump has also been studied in axons of Loligo and Sepia by Hodgkin and Keynes (1955). They found a loosely linked Na extrusion and K uptake that could be accelerated by electrical stimulation. These ionic movements were sensitive to external K but not external Na and could be abolished by metabolic inhibitors (2:4 dinitrophenol, cyanide and low temperature). This is further evidence that the external K dependent (Na)i-(K)o exchange is the active uptake of K and extusion of Na.

Since the active uptake of K and active extrusion of Na are dependent on external K, it is reasonable to expect that the inhibition of the Na/K pump by K-free Ringer will result in a loss of cellular K and an uptake of extracellular Na. This situation is (Na-enrichment, K-depletion) indeed observed in muscles that have been exposed to K-free Ringer for extended periods. For example, Steinbach (1940) and Desmedt (1953) have already been mentioned. Others include Johnson (1956), Kernan (1962), Frumento (1965) and Adrian and Slayman (1966).

The mechanism of the Na/K pump has also been extensively studied by cardiac glycosides. For example, Machett and Johnson (1954) and Johnson (1956) found that cardiac glycosides (ouabain and strophanthidin) could block the extrusion of Na and re-accumulation of K when Na-enriched K-depleted frog sartorius muscles were returned to K containing solu-

tions. Similar observations were made by Schatzmann (1953) in erythrocytes. In addition, Schatzmann demonstrated that the effects of strophanthidin were specific effects on the mechanism of ion transport and not some non-specific inhibitory effect on metabolism. The effects of cardiac glycosides [13] has been confirmed in the erythrocyte by Glynn (1957).

Since cardiac glycosides specifically inhibit the Na/K pump much like K-free Ringer albeit by an entirely different mechanism one should observe a loss of fibre K and an uptake of Na when muscles are exposed to cardiac glycosides. This is indeed observed in muscles exposed to cardiac glycosides. For example, Corrie and Bonting (1966) found that when frog sartorius muscles were exposed to cardiac glycosides for 4 hours there was a loss of intrafibre K and an uptake of Na. Clearly, both K-free Ringer and cardiac glycosides block a common mechanism of monovalent cation transport.

The location of the Na/K pump on the plasma membrane was first suggested by the work of Libet (1948) who observed a "Mg-dependent" ATPase activity in the squid axons extruded of their axoplasm. Unfortunately, the alkali cation dependence of the ATPase activity was not examined. Skou (1957)

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[13] The mechanism of inhibition of monovalent cation transport by cardiac glycosides is via the stabilization of an intermediate in the reaction sequence of the Na/K pump leading to ion transport (Skou, 1960). This inhibition is independent of energy production by the cell. Caldwell and Keynes (1960) found that only externally applied ouabain blocked ^{22}Na-efflux; the inhibition of transport was independent of ATP levels.

provided further evidence for the plasma membrane location of the Na/K pump when he found a K stimulated ATPase activity in the presence of Na and Mg in isolated fragments from finely minced crab peripheral nerves. Furthermore, this ATPase activity could be blocked by ouabain (Skou, 1960). The plasma membrane location of the Na/K pump was convincingly demonstrated by Post et al. (1960) and by Dunham and Glynn (1961). These investigators found an ATPase activity in the erythrocyte ghost which shared all the essential features of monovalent cation transport in the intact erythrocyte. Specifically, Post et al. (1960) found that: (i) both the ATPase activity and the transport required K and Na; (ii) both were inhibited by ouabain; (iii) both accepted ammonium in place of K ; and (iv) both demonstrated a competitive inhibition of K by high concentrations of external Na. Clearly, the Na/K pump is a membrane located mechanism of regulating the intracellular concentration of monovalent cations and that the normal operation of the Na/K pump is inhibited by K-free solutions and by cardiac glycosides.

The problem of heterogeneity as discussed in this thesis is not a challenge to the Na/K pump. The objective of this thesis is to point out some of the shortcomings of trying to extrapolate a membrane phenomenon to an intact cell such as a muscle fibre in which the monovalent cations are distributed heterogeneously. In fact if one is only interested in the properties of the Na/K pump alone one can dispense with

the problem of heterogeneity by using a preparation devoid of cytoplasmic structures such as the erythrocyte ghost or the squid axon with its axoplasm extruded. Alternatively, one can make use of activity measurements which measures the appropriate fraction of ions for membrane transport rather than isotope flux measurements or analytical chemistry both of which measure the cell as a whole and cannot distinguish various intracelluar compartments. Heterogeneity is important, however, if one is to consider all intracellular events during ion transport. This is especially important in experiments where the Na/K pump has been studied by perturbing the normal ionic distribution: for example by K-free Ringer or by cardiac glycosides.

Cardiac glycosides and ion-deficient Ringers have been used extensively with isotopes to dissect the various components of monovalent cation transport. The sarcolemmal fluxes of K and Na elucidated by these techniques are: (i) a (Na)i-(K)o coupled exchange blocked by cardiac glycosides which involves a (Na)i-(Na)o exchange when external K is absent; and (ii) a (Na)i-(Na)o exchange insensitive to to both external K and cardiac glycosides and saturating at a lower intrafibre Na concentration than (i). However, these experiments have, in general, not considered the effects of heterogeneity. A notable exception is Keynes and Swan (1959) who attempted to dissect the components of Na-efflux in frog skeletal muscles by following the efflux of ^{22}Na

into Na-free Ringer. They found a component of Na-efflux consistent with a (Na)i-(Na)$_o$ exchange as proposed by Ussing (1949). However, they found that if the Na-efflux were followed for more than 1 hour then the Na-efflux was consistent with the existence of a bound fraction of intrafibre Na (0.54). That is, the Na-efflux behaved as it were proportional to only 46% of the intrafibre Na. But, they argued thattheir results were also consistent with a carrier mechanism in which Na ions left the muscle fibre in groups of three. This controversy was left largely unresolved yet many subsequent investigators did not address the possible effects of heterogeneity. For example, Mullins and Frumento (1963) studied the ^{24}Na-efflux from Na-enriched muscles as a function of intrafibre Na concentration and concluded that Na ions left the muscle fibre in groups of three via a carrier mechanism. However, examination of their Figure 5 reveals that the ^{24}Na efflux is also consistent with a heterogeneous distribution of intrafibre Na. Other investigators that have not considered heterogeneity in their analyses of isotope efflux from the frog skeletal muscle include Horowicz and Gerber (1965), Keynes (1965), Sjodin and Beauge (1968 and 1973), and Horowicz et al. (1970).

The role of heterogeneity was re-considered by Keynes and Steinhardt (1968) using a two compartment model. They found that their Na-efflux was not compatible with a muscle fibre in which the Na ions were distributed homogeneously

(see their Figure 7) and debated the existence of two intrafibre compartments for Na which were either in series or in parallel with the extracellular space. Harris (1953) also reported from his labelled K fluxes that part of the intrafibre K must be bound. Unfortunately, K fluxes have not been as well documented nor as carefully studied as Na fluxes.

From the discussion above, it is clear that K-free Ringer inhibits the Na/K pump by removing the stimulatory effects of external K on the Na/K pump while cardiac glycosides inhibit the Na/K pump by stabilizing an intermediate in the reaction sequence leading to transmembrane ion transport. The overall long term effects of both treatments are a loss of intrafibre K and an uptake of Na. But, one cannot simply assume that the two treatments will have identical effects throughout the muscle fibre. For instance, K-free Ringer hyperpolarizes the membrane even after 24 hours (Lee and Armstrong, 1974) whereas a 4 hour treatment with ouabain depolarizes the membrane (Corrie and Bonting, 1966). Furthermore, what are the possible effects of the two treatments on the heterogeneous distribution of monovalent cations. The effects of solutions which perturb the normal ionic distribution on the intrafibre distribution of monovalent cations will form the main objective of this thesis.

Chapter IV

SCOPE OF THE THESIS

The main purpose of this thesis was to continue the work of Lee and Armstrong (1974) who had demonstrated that both K and Na must exist in at least two states or compartments within the frog sartorius muscle fibre and that these two ionic pools can exchange cations between each other during conditions which disturb the normal ionic distribution. Lee and Armstrong had confined their study of this problem to the new steady state resulting from long exposures to K-free Ringer (24 and 48 hours). They measured the final free and total intrafibre K an Na concentrations then by comparison with the controls deduced that both transmembrane ionic movements and internal ionic movements between the two pools had occurred.

It was elected to extend their study by examining the acute changes (within 40 minutes following a solution change) in free and total intrafibre K and Na concentrations. Furthermore, rather than confining the study to the effects of K-free Ringer , it was also elected to study the effects of ouabain (in normal Ringer), of K-and Na-free Ringer and of Na-free Ringer. In selecting the first 40 minutes for study it was my intention to capture the very

first ionic exchanges which might occur between the two internal ionic pools. There was some doubt whether what Lee and Armstrong saw after 24 hours could indeed be extrapolated back to the first hour of solution change. In selecting other perturbing solutions besides K-free Ringer, it was my initial intention only to see how intrafibre K would redistribute itself between the two internal pools when internal Na was made to increase or decrease with out necessarily altering external K.

Like Lee and Armstrong (1974) the basic experimental protocol was to measure the free intrafibre K and Na, by means of ion-selective microelectrodes, and the total intrafibre K and Na by means of atomic absorption spectrophotometry following an external solution change. In some cases the changes in free intrafibre K and Na were measured continuously following an external solution change; in most cases, however, only the initial and final free and total K and Na were determined for a given time period in a given solution. In all cases, it was essential that the microelectrode measurements were done on the same muscle and that the chemical analysis were done on paired muscles, because too much variation in electrolyte content was found, even among frogs in the same colony. Such matching of control and test muscles eventually led to a bonus during analysis. It permitted one to make meaningful comparisons of changes in experiments whose muscles might have differed signifi-

cantly in their initial absolute values for free and total K and Na. The frog skeletal muscle seemed the obvious choice for this study for the following reasons. First, it was the muscle type used by Lee and Armstrong (1974). Second, its K and Na activities had been measured by others (see Chapter VII). Third, its electrolyte content had had been measured by many others under the experimental conditions anticipated. Finally, a vast literature on the subjects of membrane ionic transport and permeability, Na pump activity, myofibrillar and sarcotubular architecture could be referred to when required. Although the basic experiment (i.e. combining ion-selective microelectrode measurements with chemical analysis) seemed relatively simple it proved rather difficult to execute. For more experiments were begun than were actually completed. The common reason for aborting an experiment included the following: (i) a dissected muscle with a relatively low initial resting membrane potentials (i.e. < 80 mV) ; (ii) an ion-selective microelectrode with deteriorating properties; and (iii) failure of the ion-selective microelectrode to recalibrate correctly at the end of an experiment.

The actual experiments successfully completed are listed here:

I. combined measurements of free and total intrafibre K and Na before and after a 40 minute (or 80 minute) superfusion with K-free Ringer;

II. a repeat of experiment I except with normal Ringer containing ouabain rather than K-free Ringer;

III. combined measurements of free and total intrafibre K and Na first following a 40 minute superfusion with K-free Ringer, then following a second superfusion with normal Ringer;

IV. combined measurements of free and total intrafibre K and Na following a 40 minute superfusion with K-free Ringer containing ouabain;

V. combined measurements of free and total intrafibre Cl following a 40 minute superfusion with K-free Ringer;

VI. measurements of intrafibre pH following a 40 minute superfusion with K-free Ringer;

VII. measurements of total Mg and Ca content following a 40 minute superfusion with either K-free Ringer or normal Ringer containing ouabain;

VIII. a repeat of experiment I except with K-and Na-free Ringer rather than K-free Ringer;

IX. a repeat of experiment I. except with Na-free Ringer (either Tris or Li substituted) as the superfuste;

X. detubulation of the muscle fibres by hypertonic glycerol shock method and verification studies to assure >90% detubulation had occurred;

XI. repeat of experiment I. except on detubulated muscle fibres.

Since the total intrafibre ionic concentrations were required, it was essential to make an extracelluar space correction on all analyzed ionic concentrations. Preliminary experiments were done to determine the extracellular space by radioactive inulin uptake method. Once a mean value was determined it was assumed to be constant and was used as a correction factor in all subsequent experiments except those involving detubulation. New inulin spaces had to be determined for the latter.

All acquired data was fitted to a two compartment model defining the distribution of intrafibre monovalent cations between one compartment measurable by the ion-selective microelectrodes and another compartment not measurable by the ion-selective microelectrodes. The mathematical derivation of this model is given in the next chapter.

Chapter V

THEORY

The concentration of an ion (as determined by chemical analysis in an excised muscle containing n compartments each having its own unique size and content, is of necessity only the average concentration for all compartments. For a muscle with n unique compartments, the total analyzed concentration, (C)t of ion C can be described as follows;

$$(C)t = \sum_{j=1}^{j=n} \alpha j\ (C)j \qquad (1)$$

where αj is the fraction of the total muscle water and (C)j is the concentration of C in compartment j.

Since the experimental data from this study only permit the analysis of ionic changes in three compartments (one extracellular and two intracellular), equation (1) must be simplified to;

$$(C)t = \alpha^{0}(C)^{0} + \alpha^{1}(C)^{1} + \alpha^{2}(C)^{2} \qquad (2)$$

where superscripts 0, 1, and 2 refer respectively to the

extracellular space, the intrafibre compartment containing free ions measured by ion-selective microelectrodes and an unknown intrafibre compartment containing the remaining C ions.

Since α° and $(C)^{\circ}$ are always known, then the total intrafibre concentration of ion C can be defined as $(C)i = (C)t - \alpha^{\circ}(C)^{\circ}$ and related to two intrafibre compartments as follows

$$(C)i = \alpha^{1}(C)^{1} + \alpha^{2}(C)^{2} \qquad (3)$$

Equation (3), in effect, defines the two compartment model used to analy ze all the experimental data of this thesis. Since most of the data analyzed represents changes in C with time, it was necessary to examine the derivative of (3),

$$\frac{d(C)i}{dt} = \underbrace{\left\{\frac{\alpha^{1}\partial(C)^{1}}{\partial t} + \frac{\alpha^{2}\partial(C)^{2}}{\partial t}\right\}}_{A} + \underbrace{\left\{\frac{(C)^{1}\partial\alpha^{1}}{\partial t} + \frac{(C)^{2}\partial\alpha^{2}}{\partial t}\right\}}_{B} \qquad (4)$$

In this equation $d(C)i/dt$ is the change in the total analyzed concentration of C in the fibre, A represents the changes in $(C)^{1}$ and $(C)^{2}$ only when ions move, and B represents changes in $(C)^{1}$ and $(C)^{2}$ only when water moves out of compartments 1 and 2. Arguments will be provided in the Results Section to discount the possibility that α^{1} and α^{2} might change signif-

icantly during the ouabain experiments, hence equation (4) can be simplified to

$$\frac{d(C)i}{dt} = \frac{\alpha^1 d(C)^1}{dt} + \frac{\alpha^2 d(C)^2}{dt} \quad (5)$$

In practice, equation (5) cannot be used for analysis since d(C)i/dt was never measured continuously. In most experiments, only initial and final determinations of (C)i and $(C)^1$ were made, hence it was necessary to restrict oneself to equation (3).

In a typical experiment, where the initial and final values of free and total intrafibre K and Na were determined equation (3) may be rewritten as follows,

$$\Delta(K)i = \alpha^1 \Delta(K)^1 + \alpha^2 \Delta(K)^2 \quad (6)$$

$$\Delta(Na)i = \alpha^1 \Delta(Na)^1 + \alpha^2 \Delta(Na)^2 \quad (7)$$

$$\alpha^1 + \alpha^2 = 1 \quad (8)$$

In equation (8) the sum of α^1 and α^2 is defined as the total intrafibre water. Since the unknowns (α^1, α^2, $\Delta(K)^2$, and $\Delta(Na)^2$) exceed the number of equations certain assumptions were necessary.

During the exposure to normal Ringer containing 10^{-4} M ouabain, it was observed that $\Delta(K)^1 > \Delta(K)i$ and that $\Delta(Na)^1 < \Delta(Na)i$ indicating that both K and Na were not uniformly distributed and that an intrafibre exchange of K and Na could be taking place. The simplest and most reasonable assumption was an electroneutral exchange of K and Na in α^2 similar to the exchange proposed by Armstrong and Lee (1971) which is diagramatically depicted in Figure 1. With this assumption then $\Delta(Na)^2 = -\Delta(K)^2$. Using this identity, equation (6) and (7) can be rewritten as

$$\alpha^1 = \frac{\Delta(K)i + \Delta(Na)i}{\Delta(K)^1 + \Delta(Na)^1} \qquad (9)$$

Implicit in this equation is the assumption that the fraction of water which acts as solvent for both free K and free Na are identical. The assumptions of electoneutrality in α^2 and identical fraction of water for free ions were maintained for all experimental conditions analyzed. Consequently, the calculated fraction of free water (α^1) and unknown water (α^2) were applied to all other experiments. Implicit in this statement is that equation (5) applies to all perturbations of the ionic distributions used in this thesis, that is, α^1, and α^2 do not change.

It should be kept in mind that the two compartment model is only a crude approximation of reality because the muscle fibre interior is known to contain more than two compartments. Furthermore, the water in each compartment may or may not act as solvent water for ions. Defined in this manner, it may appear that a calculated value for α^2 may have very little meaning. Arguments will be presented to show that most of α^2 can indeed be regarded as relatively normal mobile water belonging to one very large organelle (i.e. the myofibrils) and can account for the observed electrolyte content. Of necessity, however, it must also include water of hydration for proteins (see Chapter 1) as well as compartmentalized water in membrane limited organelles (i.e. in the sarcoplasmic reticulum, mitochondria and nuclei).

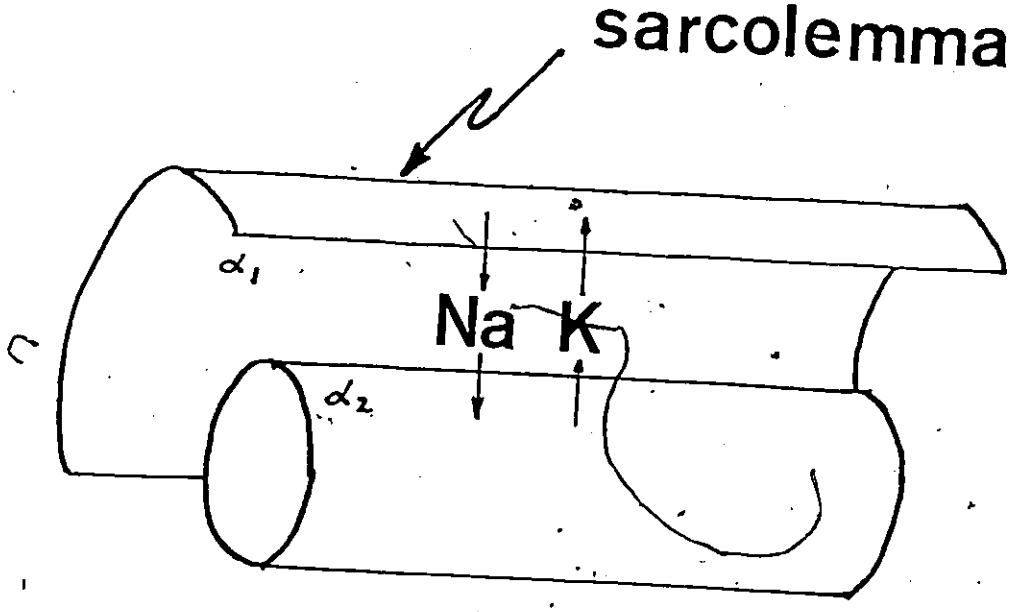

Figure 1: Schematic representation of the ionic movements in a two compartment model during exposure to normal Ringer containing 10^{-4} M ouabain. There is an influx of Na into the muscle fibre and an efflux of K out of the muscle fibre. There is an assumed 1:1 exchange of free Na for bound K in α^2.

Chapter VI

METHODOLOGY

6.1 DISSECTION AND SOLUTIONS

Sartorius muscles of Rana pipiens were used throughout. The frogs were caught at local ponds during the summer months and were purchased from various suppliers during the winter months. The frogs were pithed and both sartorii were removed as matched pairs and tied with silk threads at both tendons to a glass frame. The muscles were equilibriated in normal Ringer for 1 hour before proceeding with any experiment. One muscle of each pair was transferred to a chamber with a continuous flow of Ringer and used for microelectrode recordings while its companion muscle remained in normal Ringer. Once successful recordings were made the Ringer was changed to a solution designed to perturb the normal ionic distribution of the muscle. The recordings were either continued as the superfusate was changed or resumed after a fixed period. After the superfusion period both muscles were removed for chemical analysis.

All solutions used were 238±2 mOsm unless specified and were buffered to pH 7.2 with 10mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonicacid) from Sigma Chemical Company (St. Louis, Mo.). Normal Ringer con-

sists of 115mM NaCl, 2.5mM KCl, 1.8mM $CaCl_2$ and the buffer. K-free Ringer was made by substituting KCl with TrisCl (hydroxymethylaminomethane Cl). Ouabain containing Ringer solutions were made fresh when required by adding a pre-weighed amount of ouabain to a given volume of solution so that the final concentration was always 10^{-4} M. K-and Na-free Ringer was made by substituting the NaCl with either TrisCl or LiCl. Hypertonic glycerol Ringer was made by adding 400mM glycerol to normal Ringer.

6.2 MICROELECTRODE FABRICATION

The K-selective microelectrodes were made from Corning liquid ion-exchanger 477317 (Corning Medical, Medfeild, Mass.). Borosilicate tubing (Kimax 46485) supplied by Glass Company of America (Bargaintown, New Jersey) was pulled by a modified David Kopf 700C vertical pipette puller (David Kopf Instruments, Tujunga, Calif.) to typically give a resistance of 10-20MΩ when filled with 3M KCl. These pipettes were silanized either with Siliclad according to the protocol of Walker (1971) or with a 1% (v/v) solution of Aquasil (Pierce Chemical Co., Rockford, Ill.) in α-chloronapthalene; air dried in a dessicator for 24 hours then baked at 220 °C for 2 hours. The latter type pipette were backed filled with the ion-exchanger and a coaxial pipette filled with the reference solution was lowered into the ion-exchanger (Ujec et al., 1979).

The Cl-selective microelectrodes were manufactured in the same manner as the K-selective microelectrodes except by using Corning liquid ion-exchanger 477315. The reference solution was 0.5M KCl for both the K and Cl-selective microelectrode. The configuration of the Walker electrodes and the Ujec electrodes are shown in Figure 2.

The Na-selective microelectrodes and pH microelectrodes were of the Thomas configuration (Thomas, 1970). The configuration of the electrodes are shown in Figure 3. The inner blind capillary of Na-selective microelectrodes was made from Na-selective glass (Corning NAS_{11-18}) and the pH microelectrodes were constructed from pH glass (Corning 0150). The NAS_{11-18} was pulled from pieces of 1.0mm outer diameter (O.D.) tubing on the David Kopf 700C puller by using 22 gauge wire made of 90% Pt /10% Ir (Englehardt Industries, Toronto, Ont.); whereas the pH glass was pulled from 1.0mm O.D. pieces using 26 gauge Nichrome wire (Englehardt Industries, Toronto, Ont.).

The tips of the resulting pipettes of ion-selective glass were sealed by means of a home brewed microforge which made use of a piece of 25 um Pt wire to form a sealed membrane. It is important to minimize the heat used in working with the Na-selective glass since excessive heating at any phase of the fabrication will diminish the selectivity towards K. The sealed pipettes were then manipulated into the insulating micropipette made from aluminosilicate glass tubing

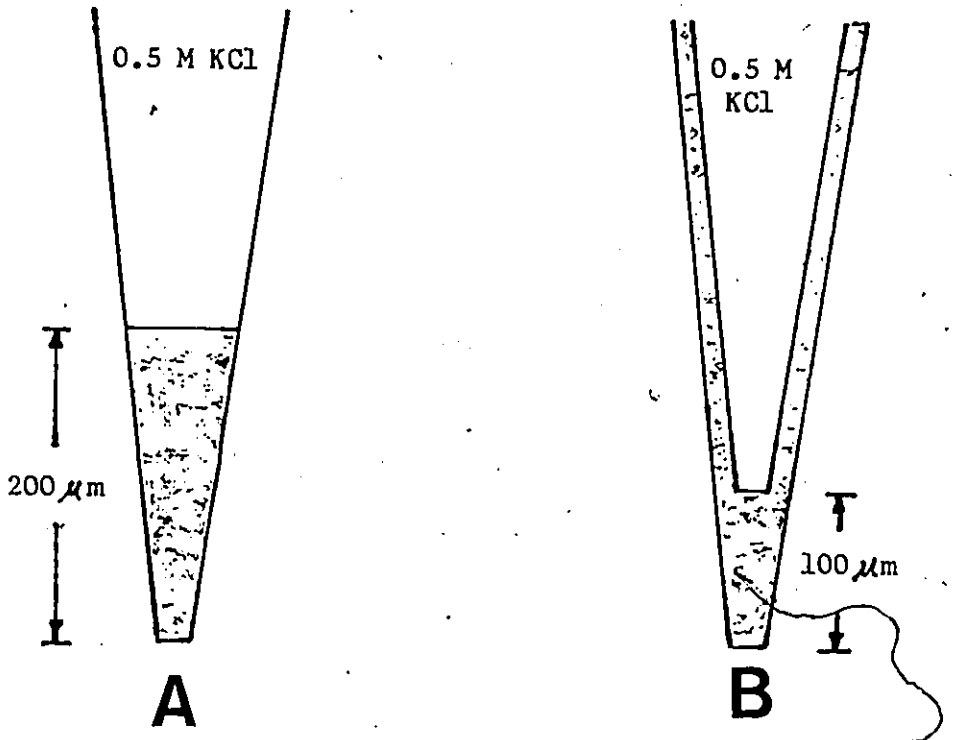

Figure 2: The configuration of the Walker type of liquid ion-exchange electrodes (A) and of the Ujec type of liquid ion-exchange electrodes (B). Both types of electrodes were silanized by dipping their tips into a silane solution (see text). The Walker type was filled with liquid ion-exchanger by dipping their tips into a pool of liquid ion-exchanger and allowed to fill by capillary acton. The reference solution (0.5 M KCl) was injected into the microelectrode barrel. The Ujec type was filled with liquid ion-exchanger in an identical manner, however, an additional amount of ion-exchanger was back injected into the microelectrode barrelto form an ion-echange column of about 5mm. A coaxial microelectrode filled with the reference solution was advanced into the liquid ion-exchange phase. The tips are not drawn to scale.

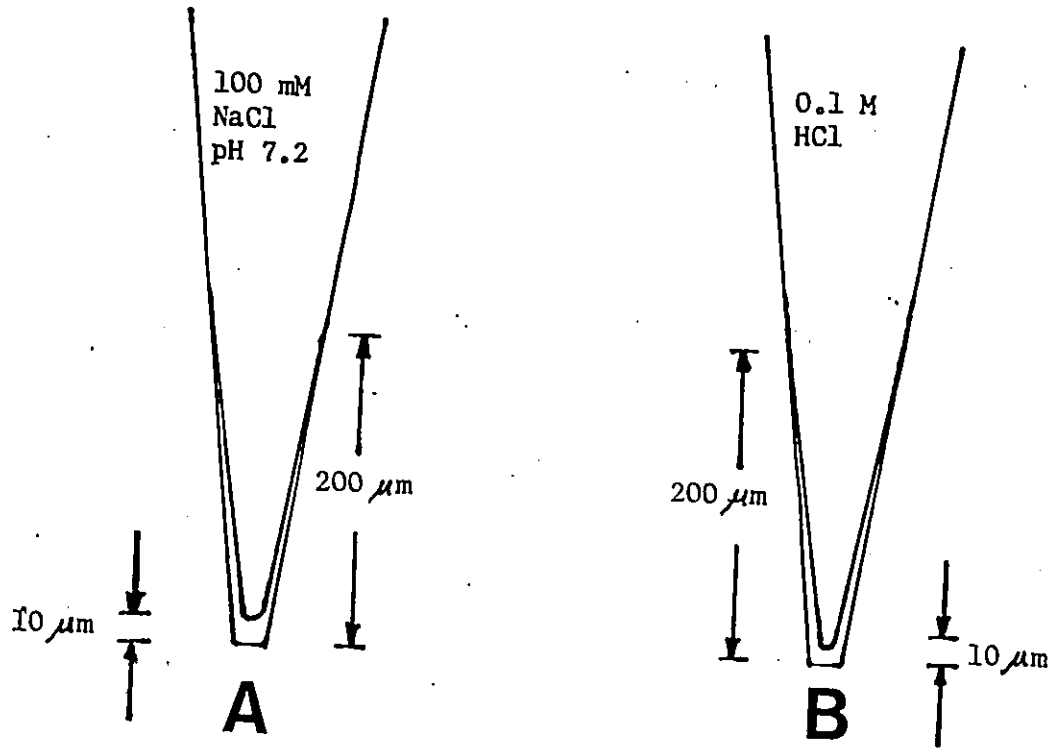

Figure 3: The configuration of the Thomas type of Na-selective micrbelectrode (A) and pH-microelecrode(B). The outer insulating micropipette of both electrode types were fabricated from aluminosilicate tubing and was sealed to the ion-selective glass at approximately 200 um from the tips as shown. The tips are not drawn to scale.

(Glass Company of America, Bargaintown, N.J.). Aluminosili-

cate was chosen because it has the highest specific resistivity and for its resistance against tip breakage. Typically, the aluminosilicate pipettes had resistances of 20-40 M when filled with 3M KCl. The Na-selective glass and pH glass were then sealed to the insulating pipette by the microforge to the as described by Thomas (1970). The completed microelectrodes were stored in a dessicator until use. The Na-selective microelectrodes were filled with 100mM NaCl buffered to pH 7.0 with 10mM Hepes. The pH microelectrodes were filled with either 0.1M HCl or 100mM NaCl buffered to pH 7.0 with 10mM Hepes.

The open tipped microelectrodes were made by pulling micropipettes from Kwik fill tubing from WPI or from Glass Company of America on the David Kopf 700C. The micropipettes were filled with 3M KCl.

6.3 MICROELECTRODE CALIBRATION AND REJECTION CRITERIA

Ion-selective microelectrodes are not perfectly specific to one ionic species, that is, they respond to other ions. The equation which describes the behavior of an ion-selective microelectrode is

$$E = E^{\circ} + \frac{RT}{zF} \log\left(a_i + \sum_{j \neq i} k_{ij} a_j^{z_i/z_j} \right) \qquad (1)$$

where

E is the measured potential, E° is the electrode potential when $(a_i + \sum_{j \neq i} k_{ij}a_j = 1)$, a is the ionic activity of ion i (The principle ion), a is the ionic activity of ion j (the interfering ion), k is the selectivity co-efficient of the electrode towards ion j relative to ion i, and RT/zF has its usual thermodynamic significance.

Under the experimental conditions of this thesis, only one interfering ion had to be considered per microelectrode type. For monovalent ions equation (1) simplifies to

$$E = E^\circ + \frac{RT}{zF} \log(a_i + k_{ij}a_j^{z_i/z_j}) \qquad (2)$$

The ion-selective microelectrodes do not respond with an ideal slope of 2.3 RT/zF for a ten fold change in activity and for practical purposes equation (2) simplifies to

$$E = E' + S \log(a_i + k_{ij}a_j) \qquad (3)$$

for monovalent ions where E', S and k_{ij} are constants for a given electrode determined from a set of standard solutions containing known activities of ion i and ion j.

For this thesis, electrodes chosen for actual experiments were such that the interfering ions under the experimental conditions did not cause significant errors in the determi-

nation of the activity of the principle ion (i.e. $k_{ij}a_j$ is negligible). Equation (3) then simplifies to

$$E = E' + S \log(a_i) \qquad (4)$$

The slope, S, of the K-selective microelectrode was determined in standard solutions containing 10mM and 100mM KCl. The selectivity co-efficient over Na was determined by comparison with a standard solution containing 50mM KCl + 50mM NaCl.

The slope of the Cl-selective microelectrode was determined in standard solutions of 1mM, 10mM and 100mM KCl. The Cl-selective microelectrode has a relatively poor selectivity for Cl over HCO_3. However, under the experimental conditions the intrafibre HCO_3 level was low and will not cause a significant error in the Cl activity determination. Nevertheless, Cl:HCO_3 selectivity curves will be shown in Appendix A.

All ion-exchange microelectrodes used had input resistances of 10^9-$10^{11}\Omega$. The K-selective microelectrode was rejected if : (i) the 90% response time from 10mM KCl to 100mM KCl exceeded 2 seconds; (ii) S < 55mV between 10mM KCl and 100mM KCl; and (iii) if $k_{K,Na} > 0.01$. The Cl-selective microelectrodes were rejected if : (i) the 90% response time from 10mM to 100mM KCl exceed five seconds; and (ii) S < 55mV between 10mM KCl and 100mM KCl.

The slope of the Na-selective microelectrode was determined in 10mM NaCl and 100mM NaCl buffered to pH 7.2 with 1mM Hepes. The selectivity co-efficient for Na over K was determined by comparison with standard solutions containing 10mM NaCl + 90mM KCl and 50mM NaCl + 50mM KCl. The input resistance of the Na-selective microelectrode was typically 10^{10}-$10^{11}\Omega$. The electrodes were rejected if : (i) the 90% response time from 10mM KCl and 100mM NaCl exceeded 1 minute; (ii) S < 55mV between 10mM NaCl and 100mM NaCl; and (iii) if $k_{Na,K} > 0.01$.

The pH microeletrode was calibrated in standard commercial buffers ranging from pH 6.0 to 8.0. These electrodes were rejected if: (i) the 90% for a decrease of 1pH unit, exceeded 1 minute and (ii) S<55mV. The input resistance was typically 10^{10}-$10^{11}\Omega$.

The open-tipped microelectrodes were routinely checked in 200mM KCL and 200mM NaCl. The input resistance was typically 20-40MΩ. These electrodes were rejected if: (i) the junction potential in 200mM KCl differed by more that 1mV from that in 200mM NaCl; and (ii) the noise exceeded 1mV peak to peak.

6.4 RECORDING APPARATUS

The recording apparatus is shown in Figure 4. Because of the high impedence of the ion-selective microelectrodes it was necessary to place this apparatus in a Farady cage.

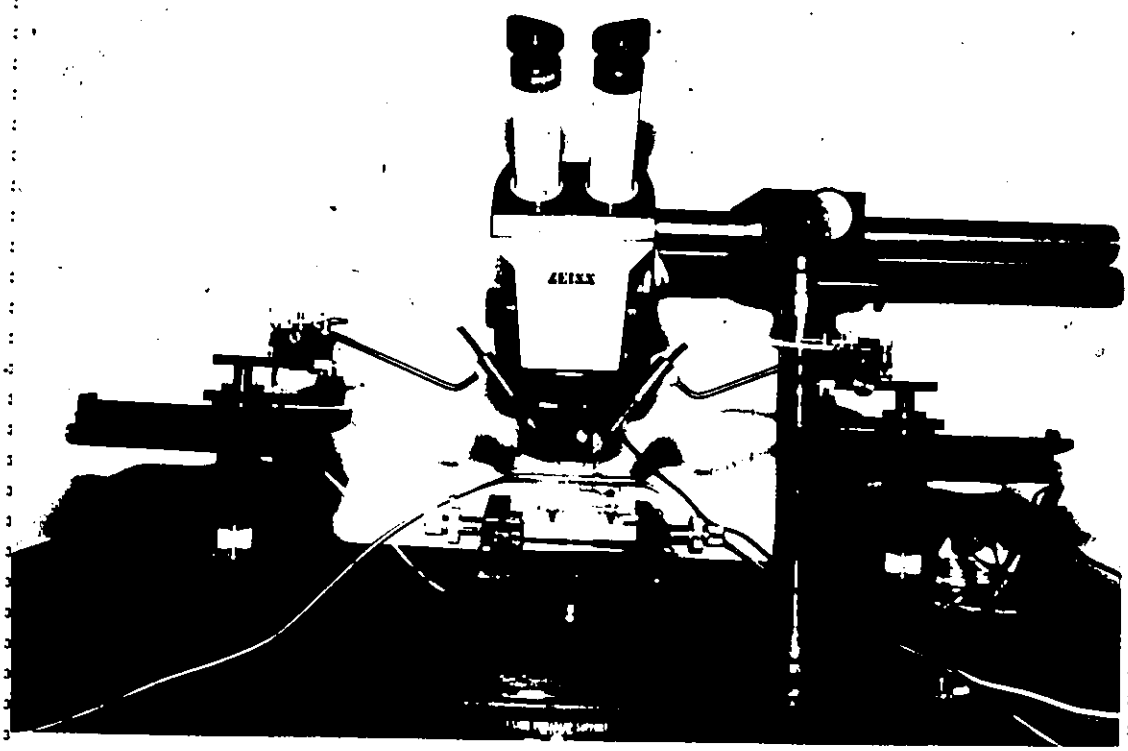

Figure 4: Photograph of the microelectrode recording apparartus and the the recording chamber in experimental position. The recording chamber is connected to a push pull pump which maintains a fresh supply of solution to the muscle at all times. The microelectrodes are mounted directly onto the preamplifiers of the electrometer. The microelectrode and the preamplifier are moved as a unit by the micromanipulators. The recording chamber and the micromanipulators are floated on a Micro-g Table and confined to a Faraday Cage.

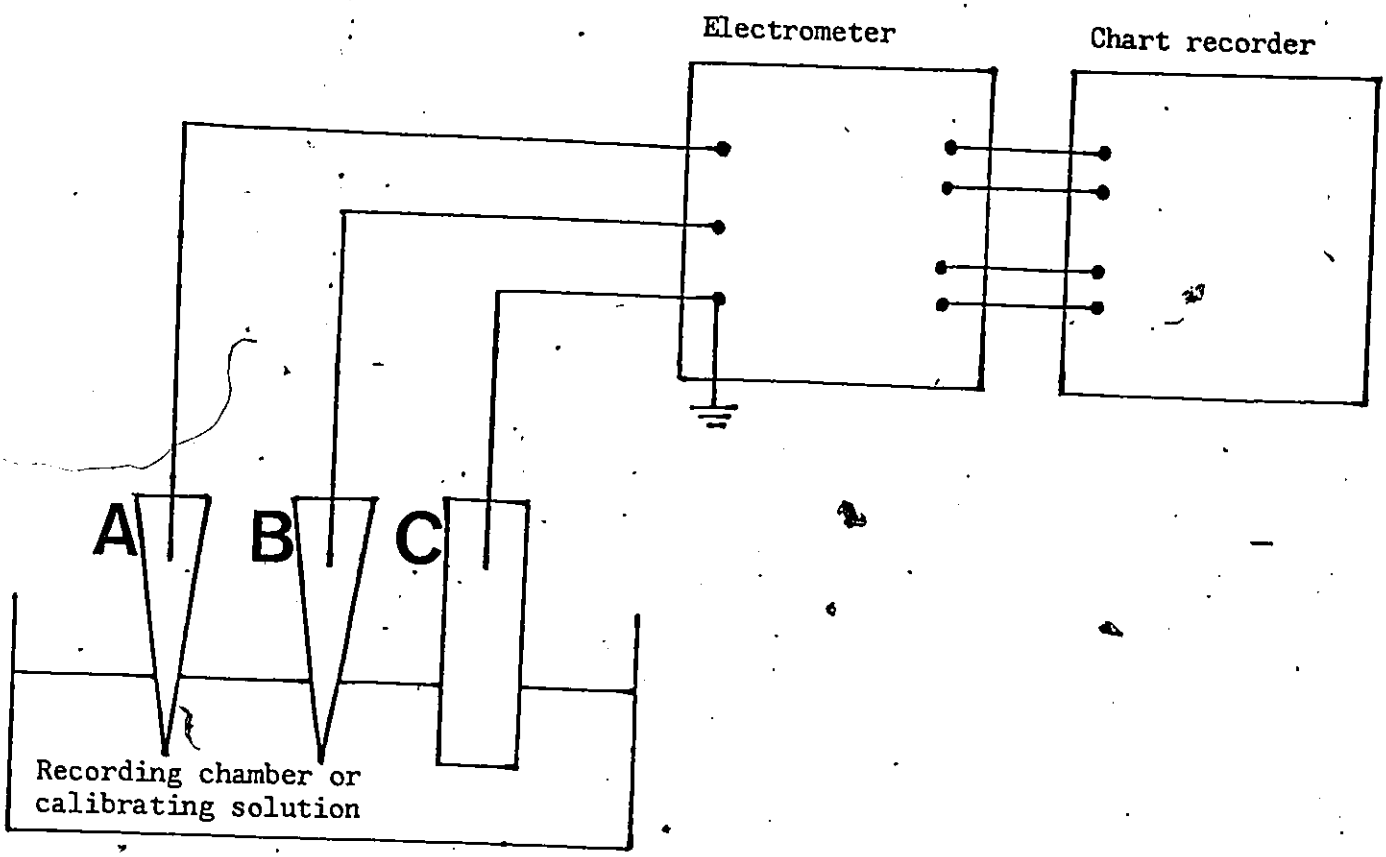

Figure 5: Schematic diagram of the recording apparatus. Electrode A is the ion-selective microelectrode, electrode B is the open tipped microelectrode, and electrode C is the calomel electrode. Both microelectrode can be referred to the calomel electrode and electrode A can be referred to electrode B (see text). The output of the electrometer is connected to the chart recorder for a hard copy of the data. This apparatus was used in both the actual experiments and in the electrode calibration.

The recording apparatus is schematically represented in Figure 5. It consists of a commercial calomel electrode in contact with the bathing solution (or a calibration solution) through a saturated KCl bridge to which microelectrodes can be referred to. The electrodes (microelectrode and calomel) are connected to a WPI FD-223R electrometer (see Figures 4 and 5). This circuit was used for both intracellular recordings and electrode calibrations.

The ion-selective microelectrodes when pushed into a muscle fibre measures a sum of two potentials, one associated with the intrafibre ionic activity, Ei, and the membrane potential, Em. That is,

$$E = Ei + Em \qquad (5)$$

where E is the measured intrafibre potential. The WPI electrometer allowsfor the simultaneous measurement of Em (the open-tipped microelectrode is referred to the calomel electrode) and the subtraction of Em from E (the ion-selective microelectrode is referred to the open-tipped microelectrode). Ei and Em were then recorded on an Esterline Angus Speed Servo II strip chart recorder (Indianapolis, Ind.). The electrometer and the chart recorder were calibrated biweekly against a standard voltage source.

For intrafibre recordings, the bath chamber in Figure 6 was used. The shaded portion of this chamber is filled with

Sylgard to facilitate pinning of the silk threads tied to the muscle tendon. The raised central portion contains two pieces of 22 guage Pt wire. These wires serve a dual purpose of raising the central portion of the muscle to facilitate microelectrode punctures and to ensure that the muscle is exposed to fresh solution on both surfaces and of acting as stimulating electrodes.

The muscle was pinned in the chamber so that fibres on its deep surface (in situ) were used for the microelectrode measurements. The chamber was connected to a pump which simultaneously drives solution into and out of the chamber while microelectrode recordings were in progress.

The total volume of the chamber is 3.0ml, but, during an experiment the tyical working volume was 1.5ml. The flow rate while recordings were in progress was 0.5ml/min except during a solution change when the rate was increased to 2.0ml/min. At this rate the solution within the bath could be changed in about 2 minutes without disturbing recordings in progress.

The calibration of the microelectrodes were performed using beakers filled with the calibration solutions.

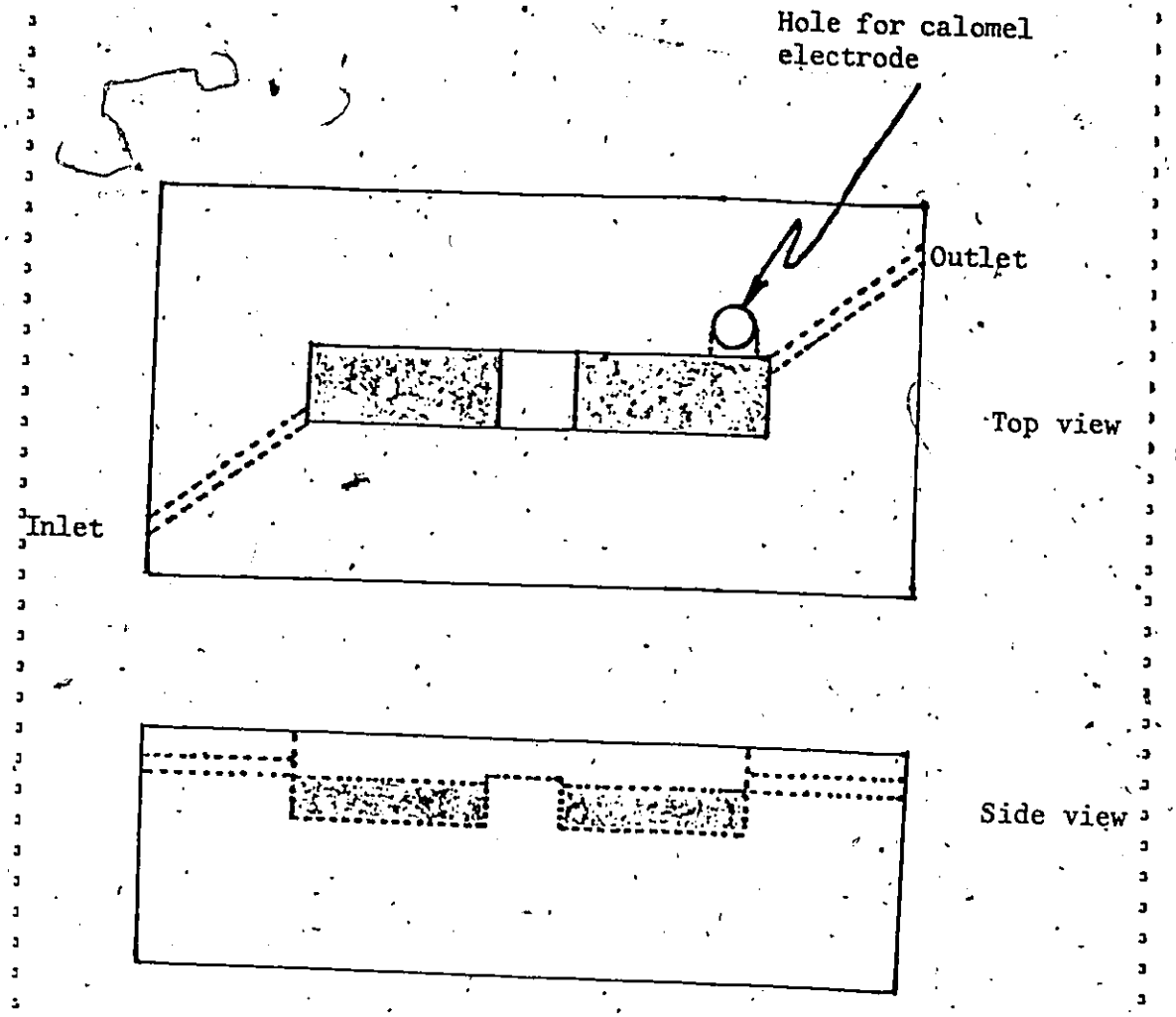

Figure 6: Scaled drawing (1X) of the recording chamber. The chamber was milled from a single piece of plexiglass. The shaded area is filled with Sylgard for pinning the muscle. The raised central portion contains two 22 gauge Pt wires (not shown) which serves the dual purpose of supporting the muscle and acting as stimulating electrodes when required.

6.5 CRITERIA FOR REJECTING AN EXPERIMENT

All microelectrodes used for an actual experiment were calibrated before and after an experiment. The experiment was discarded if the calibration of any of the microelectrodes in any one standard solution deviated by more than 1mV before and after an experiment. This criterion was chosen because even an error of 1mV in either the K-selective microelectrode will cause a relatively large error in the calculated $(aK)_i$.

Single muscle fibres were punctured by both an ion-selective microelectrode and an open-tipped microelectrode. The electrode puncture sites were typically seperated by about 250 um. The sequence of punctures with the electrodes was varied with either electrode being first.

Two types of recordings were performed in this thesis. In one group the recordings were continuous. Single surface fibres were punctured in normal Ringer. Once a stable recording was acheived the superfusing solution was changed to a solution which perturbed the normal ionic distribution. The second group of experiments were samplings. Recordings were made from several surface fibres in normal Ringer. These recordings were resumed usually after 40 minutes and were not extended past ten minutes of the designated time. These recordings were accepted only if recordings from both electrodes were stable to within 1mV for 1 minute.

Intrafibre ionic activities were calculated according to equation (4).

6.6 DETERMINATION OF FREE IONIC CONCENTRATION FROM IONIC ACTIVITY

According to the Debye-Huckel theory concentration is related to activity by ;

$$a^i = \gamma_{\pm} C^i \qquad (5)$$

where a^i is the activity of ion i, C^i is the analytical concentration of ion i, and $\gamma_{\pm}$ is the mean activity coeffient for both the cation and anion of the salt. Emperically, a^i can be considered as the effective thermodynamic concentration of ion i. Thus $\gamma_{\pm}$ is a co-efficient which relates the interaction of ion i with other molecular species. The interactions usually lowers the effective concentration from the analytical concentration[14]. A detailed treatment of the Debye-Huckel theory is well documented and unnecessary to this thesis. The interested reader is referred to any standard textbook of electrochemistry. Briefly, according to the Debye-Huckel Theory

[14] As ionic strength increases $\gamma_{\pm}$ can exceed unity for some solutes, for example KOH.

$$\log \gamma_{\pm} = \frac{A|Z_+Z_-|I^{\frac{1}{2}}}{1 + BaI^{\frac{1}{2}}} + bI \qquad (6)$$

where A, B, a, and b are constants, Z_+ is the charge of the cation in solution, Z_- is the charge of the anion solution, and I is the ionic strength of the solution defined as

$$1/2 \sum_i C^i z^i \qquad (7)$$

where C^i is the concentration of ion i and z^i is the valence of ion i.

Since the mean activity co-efficient for the mixture of ions in the myoplasm cannot be measured, it has become convention to regard it as equal to the extrafibre activity co-efficient ($\gamma_{\pm}^{o}$), that is, $\gamma_{\pm}^{o} = \gamma_{\pm}^{i}$. In support of this assumption Hinke (1970) deduced from single barnacle muscle fibres that the intrafibre activity co-efficient was about 0.64 which closely agrees with the activity co-efficient of barnacle Ringer (0.65). Since $\gamma_{\pm}^{o}$ for frog Ringer is 0.75, this thesis continues the convention of assigning the same value to $\gamma_{\pm}^{i}$ of the frog myoplasm.

6.7 DETERMINATION OF TOTAL INTRAFIBRE IONIC CONCENTRATION

Chemical analysis was always performed on paired muscles. One muscle of the pair was exposed to a solution designed to disturb its electrolyte distribution while its companion remained in normal Ringer and served as a control. This feature was essential in order to properly document the effects of the experimental solutions on the changes in the electrolyte distribution, since the electrolyte content were found to vary widely from frog to frog.

All muscles used for chemical analysis were blotted on Whatman 2 filter paper in an identical manner and placed in stoppered preweighed borosilicate vials. The muscle wet weights were determined. The muscles were then oven dried overnight at 95 °C. The dry weights were then determined and the muscle water content was calculated as the difference between the wet and dry weights.

Muscles analyzed for K and Na were digested in concentrated nitric acid then neutralized with ammonium. The digests were then transferred to volumetric flasks and made up to volume. The K and Na contents were then determined by atomic absorption spectroscopy on a Varian AA-5 atomic absorption spectrophotometer (Varian Techtron, Melbourne, Austrailia). The total muscle K and Na concentrations were calculated from the analytically determined total K and Na and the muscle water content.

Muscles analyzed for Cl were digested in NaOH and processed according to the procedure of Cotlove (1964). The Cl content of the digests were then determined by electrometric titration on a Buchler-Cotlove chloridometer (Buchler Instruments, Fort Lee, N.J.). The total muscle Cl concentration was calculated from the analytically determined total Cl and the muscle water content.

Muscles analyzed for Ca and Mg were digested with a solution of $LaCl_3$ and perchloric acid according to the procedure of Ueba et al. (1971). The digests were then transferred to volumetric flasks and made up to volume. The Ca and Mg contents were then determined by atomic absorption spectroscopy. The total muscle Ca and Mg concentrations were calculated from the analytically determined total Ca and Mg and the muscle water content.

The total intrafibre concentration of ions were calculated from the total muscle concentration minus the extracellular space contribution.

Before the total intrafibre concentration of an ion could be obtained it was necessary to know the size and ionic content of the extracellular space (see theory section). This was determined by ^{14}C-inulin uptake. Commercial ^{14}C-inulin (New England Nuclear, Lachine, Quebec), upon arrival was filtered through a UM05 DIA FLO filter (Diamed Lab SUpplies, Mississauga, Ont.) in order to obtain a population of inulin molecules in excess of 500 daltons. The unfiltered residue

was dissolved in normal Ringer and used in the following manner.

Muscles were exposed to normal Ringer containing ^{14}C-inulin for 2 hours unless specified and blotted in exactly the same manner as muscles use for chemical analysis. The muscle wet weight, dry weight and water content were determined as described above. The dried muscles were digested in Soluene-350 (New England Nuclear) activated with water at 70 °C overnight. The digests were then treated with 30-35% H_2O_2 to remove color quenching, then treated with glacial acetic acid to remove chemiluminescence. The samples are then dissolved in Aquasol (New England Nuclear) and counted on a Beckman LS 9000 liquid scintillation counter (Beckman Instruments, Irvine, California). Each sample was counted several times until stable counts were acheived. This step was essential to ensure that spurious counts from chemiluminescence or from photoluminescence did not contribute to the calculated ^{14}C-inulin content.

The extracellular space was calculated with the assumption that inulin did not enter the muscle fibre. The extracellular space was calculated as a fraction of the muscle water content, hence

$$\alpha^{\circ} = \frac{C^{1}}{MxC^{\circ}} \qquad (8)$$

where α° is the extracellular space expressed as a fraction of the total muscle water content, C^{1} is the experimentally determined muscle ^{14}C-inulin content, M is the total muscle water content, and C° is the concentration of ^{14}C-inulin in the bath. The total intrafibre concentration of an ion was then calculated from the relation

$$(I)i = \frac{I^{1} - \alpha^{\circ}I^{\circ}}{1 - \alpha^{\circ}} \qquad (9)$$

where (I)i is the calculated total intrafibre concentration of ion I, I^{1} is the experimentally determined total muscle concentration of ion I, α° is the fractional volume of the extracellular space, and I° is the concentration of ion I in the bath.

6.8 TYPICAL PROTOCOL

Paired muacles were used throughout unless specified. In a typical experiment, one muscle of each pair is transferred to the recording chamber where microelectrode recordings were made. The solution was then changed and more recordings were made. Meanwhile the companion muscle is maintained in normal Ringer. At the end of the recording period both muscles were removed for chemical analysis.

Chapter VII

(VII) SEASONAL VARIATION OF INTRAFIBRE K-LEVELS

Experiments were performed year round from 1979 to 1983 on sartorius muscles of Rana pipiens from various sources as outlined in the Methodology Section. The intrafibre K activity, $(aK)^i$, and the total intrafibre K concentration, $(K)i$, were observed to vary with season. The data are summarized in Table 1 grouped arbitrarily by season.

Notice that $(aK)^i$ was lowest and $(K)i$ was highest during the winter months. The data indicated that winter frogs have the highest intrafibre K content but that a larger fraction of it is bound while the converse holds for summer frogs. However, a rigorous comparison of any of the parameters cannot be made since the frogs were not obtained from the same source. The summer frogs were generally the healthiest, the most active and survived the longest in captivity

The overall average value of $(aK)^i$ reported in this thesis is higher than those reported by Walker and Brown (1977). This discrepancy is worthy of comments. Their values of $(aK)^i$ range from 91×10^{-3} to 105×10^{-3} with an average of 97×10^{-3}. The data of Table 1 are summarized in Table 2 along with previously published K measurements. The most

Table 1: SEASONAL VARIATION OF K-LEVELS IN FROG SARTORIUS MUSCLES IN NORMAL RINGER

	RESTING POTENTIAL Em(mV)	FREE K $(aK)^{1} \times 10^{-3}$	TOTAL K‘ (K)i (m.moles/kg f. w.)
WINTER	-85±0.7(95)	101±2.0(85)	150±1.5(96)
	p=0.02	p<0.01	p=0.035
SPRING	-87±0.5(104)	112±2.0(60)	145±1.8(63)
	p=0.01	p=0.01	p=0.065
SUMMER	-89±0.5(167)	131±5.0(75)	140±2.0(65)

All values are mean ± 1 standard error, the numbers in brackets are the number of determinations.

‘ Corrected for the extracellular space using a value of 20% of the muscle water.

p is the probability that the mean above and below are equal.

interesting feature of this table is that the calculated K equilibrium potential Ek, is relatively constant ranging from -99mV to -101mV, yet the measured Em varies from -82 to -92 mV (the values of this thesis were not included). This indicated that perhaps there are variations of the resting K permeability and/or Na permeability that have not been previously documented. Notice also that the difference between

Em and Ek for isolated muscles remains relatively constant ranging from -15mV to -19mV with an average of -17mV (the values of Kernan and MacDermott were not included).

Table 2: DATA OF $(aK)^1$, $(K)i$, Em AND Ek FROM FIVE PREVIOUS STUDIES AND FROM THIS THESIS.

	$(aK)^1$ $x10^{-3}$	$(K)i$ (mM)	Em(mV)	$(aK)^6$ $x10^{-3}$	Ek (mV)	Ek - Em (mV)
Lev[4]	98	127	-82	1.9	-101	-19
	96	124	-82	1.9	-100	-18
Kostyuk et al.[4]	94	133	-83	1.9	-100	-17
	96[9]	134	-86	1.9	-100	-14
Khuri et al.	105[9]	142	-89	2.6	-94	-5
Lee and Armstrong	94	140	-85	1.9	-100	-15
	95	142	-89	1.9	-100	-15
	97	141	-85	1.9	-101	-16
Kernan and MacDermott[6]	91	128	-92	1.9	-99	-7
Winter	101	150	-85	1.9	-102	-17
Spring	112	145	-87	1.9	-104	-17
Summer	131	140	-89	1.9	-108	-19

See bibliography of this chapter for references

[4] Superfusing solution only given as Ringer. $(aK)^6$ assumed to be 1.9.

[9] In situ muscles.

This difference does not seem to vary with season. There are two other interesting features of Table 2. First, the membrane potential (data from this thesis), varies with the seasonal variation of $(aK)^1$ (i.e. the membrane behaves as a as a K-electrode). Second, the variation in (K)i but not in $(aK)^1$ indicates that there may also be variations in the fraction of free and bound K not previously documented. However, the variations in (K)i may be artefactual. For example, Lev (1964) used an extracellular space of 10% to calculate his (K)i. If an extracellular space of 20% was used instead, his (K)i would be about 140 m.moles/kg fibre water. Unfortunately, this contention cannot be tested with the data from the other investigators since their values for the extracellular space were not given with the exception of Lee and Armstrong (1974). They found an (K)i of about 140m.moles/kg fibre water using an extracellular space of about 20%. It is also interesting that the value of $(aK)^1$ and Em from winter frogs are in close agreement with those of Lee and Armstrong (1974) who also used Rana pipiens. However, the spring and summer intrafibre K activities were higher and Em was more negative accordingly.

There were no seasonal variations in either the intafibre Na activity, $(aNa)^1$, or the total intrafibre Na concentration, (Na)i. The overall mean water content was 78.5% of the muscle wet weight with no seasonal variation. In all muscles (Na)i was always greater than $(aNa)^1$ indicating

binding of intrafibre Na. Variations in $(aNa)^1$ and (Na)i were correlated with the state of health of the individual animals, both parameters increasing in sickly animals. The data from sickly frogs were included if their resting membrane potential exceeded -80 mV as described in the Methodology section. In healthy frogs $(aNa)^1$ was about 10×10^{-3} while (Na)i was about 30m.moles/kg fibre water. In isolated muscles, $(aNa)^1$ has been reported to be about 9×10^{-3} (Kostyuk et al., 1969) to as low as 5×10^{-3} (Lev, 1964).

Clearly, these observations illustrate the importance of using a stringent set of controls when one is examining changes in the electrolyte content of frog skeletal muscles. In general, there were variations from frog to frog and from batch to batch but not within an animal. In all experiments presented in this thesis, activity measurements during a change in the experimental conditions are only referred to its control measurements in the same muscle, while total intrafibre concentrations are only referred to its companion muscle stored in normal Ringer. The experiments were discarded if the activity measurements were not successful in both the control and the experimental condition or if there was damage to either muscle of a pair during the handling for chemical analysis.

Chapter VIII

THE DIFFERENTIAL EFFECTS OF K-FREE RINGER AND OUABAIN ON THE INTRAFIBRE ELECTROLYTE DISTRIBUTION

8.1 INTRODUCTION

As discussed in Chapter III, K-free Ringer and ouabain have been used to study the Na/K pump. This chapter examines the effects of K-free Ringer and ouabain on the intrafibre distribution of K and Na during the first hour of treatment. The data is analyzed by the two compartment model of Chapter IV. It will become apparent that the acute exposures to K-free Ringer and ouabain produce markedly different alterations to the intrafibre distribution of K and Na.

8.2 RESULTS

8.2.1 CONTINUOUS RECORDINGS OF $(aK)^1$ AND $(aNa)^1$ WHEN NORMAL RINGER IS REPLACED BY K-FREE RINGER.

In this group of experiments single sartorius muscles were used. $(aK)^1$ or $(aNa)^1$ and Em were recorded continously from a single surface fibre before superfusion with K-free Ringer and, for 40 minutes after the onset of superfusion with K-free Ringer.

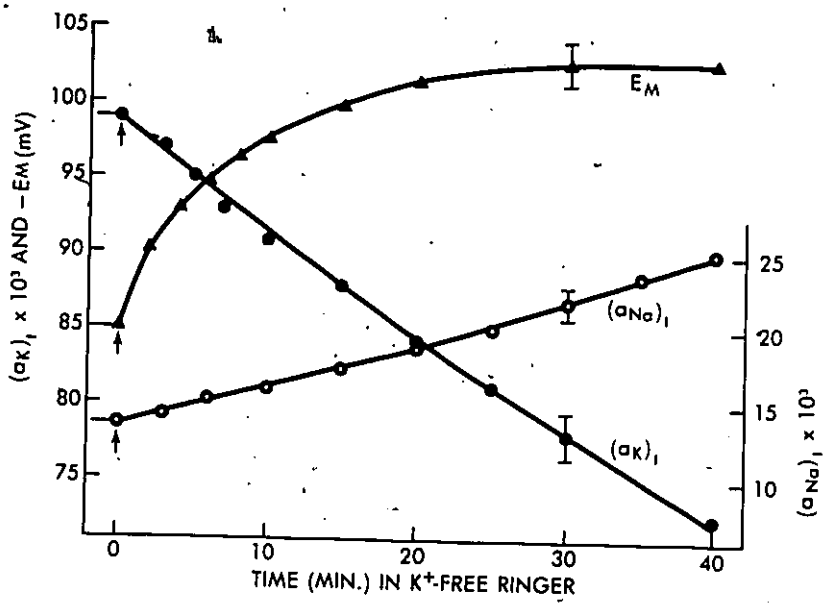

Figure 7: The averaged continuous recordings of $(aK)^1$ (11 experiments), $(aNa)^1$ (9 experiments) and Em (20 experiments) when the external superfusate was changed from normal Ringer to K-free Ringer (at arrow). The bars at 30 minutes are ± 1 standard error of the mean.

Figure 7 summarizes the averaged recordings of $(aK)^1$, $(aNa)^1$ and Em. There was a consistent and significant fall

in $(aK)^1$ and a consistent and significant increase in $(aNa)^1$. These free ionic changes are in qualitative agreement with what should occur when the Na pump is inhibited, that is, a loss of K and a gain of Na. However, $(aK)^1$ fell much more rapidly than $(aNa)^1$ increased. The fall of $(aK)^1$ can be fitted by a single exponential with a rate constant of 0.48 hr^{-1}. This rate is approximately 8 times the rate constant for K-efflux measured by isotopes under similar conditions (Mullins and Noda 1963 Sjodin and Henderson, 1964; Henderson, 1971b; and Sjodin and Beauge, (1973).

It could be argued that $(aK)^1$ was measured in surface fibres while the isotope fluxes were measured in whole muscles and that the discrepancy is caused by delays in the extracellular space of whole muscles. Arguments will be given below to discount this contention. It is worth mentioning though that the ion-selective microelectrode only provides information about the free fraction of ions, and not ionic changes in the whole fibre. These activity measurements must be supplemented by total chemical analysis to properly examine the problem of heterogeneity.

Figure 1 also reveals that hyperpolarization of the membrane potential accompanied the fall in $(aK)^1$. This has significance because it indicates that the original 2.5mM of extracellular K must have been adequately removed during K-free Ringer superfusion. It also suggests that the lost extracellular K might not have been replenished by transmembrane movements of K even though $(aK)^1$ was observed to fall.

8.2.2 CHANGES IN THE FREE AND TOTAL K AND Na DURING A 40 MINUTE EXPOSURE TO K-FREE RINGER

This set of experiments combined the microelectrode measurements with total chemical analysis to compare the changes in free K and Na with the changes in total intrafibre K and Na. Details of the procedure have been described in the Methods, and the mean results from successfully completed experiments are given in Table 3.

The changes in the free K concentration [15], $(K)^1$, and the free Na concentration, $(Na)^1$, are in agreement with those of Figure 1, that is, a large drop in $(K)^1$ of -36mM and an increase in $(Na)^1$ of +16mM. The table also shows that the total intrafibre K concentration, (K)i, was unchanged while the total intrafibre Na concentration, (Na)i, increased 5.5m.moles/kg fibre water. There was no detectable change in the muscle water content of treated muscles relative to their controls.

The apparent constant level of total intrafibre K concentration is in agreement with Adrian (1956) who measured (K)i by flame photometry and found no significant change in muscle content after a one hour equilibration in K-free Ringer. Indeed Simon et al. (1957) found that toad sartorius muscles maintained their total K in K-free Ringer for up to 4 hours.

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[15] The free concentrations were calculated by dividing the activity measurements by an activity co-efficient of 0.75 as detailed in the Methodology Section.

Table 3: CHANGES IN FREE AND TOTAL K AND Na BEFORE AND AFTER A 40 MINUTE SUPERFUSION WITH K-FREE RINGER

Solution	Em(mV)	FREE CONCENTRATION[3] (K)i (mM)	FREE CONCENTRATION[3] (Na)i (mM)	TOTAL CONCENTRATION[4] (K)i (m.moles/kg. f.w.)	TOTAL CONCENTRATION[4] (Na)i (m.moles/kg. f.w.)
Normal Ringer	-85±0.8 (20)	133±0.5 (12)	16±1.2 (8)	151±2.0 (20)	22±1.1 (20)
K-free Ringer	-103±1.4 (20)	97±1.7 (12)	32±1.2 (8)	150±1.8 (20)	27.5±1.2 (20)
Difference	-18±1.6 (38) p<0.001	-36±1.8 (36) p<0.001	+16±1.7 (14) p<0.001	-1±2.7 (38)	+5.5±1.6 (18) p<0.002

All values are mean ± 1 standard error, the numbers in brackets are the number of determinations.

[3] Obtained by dividing the K and Na activities by an assumed mean activity co-efficient of 0.75.

[4] Values are corrected for an extracellular space of 20% of muscle water. The p values are the probabilities that the differences are zero (student t-test).

A constant total intrafibre K content indicates that the observed fall in free K cannot be related to transmembrane movement of K. Instead, it suggests either that free K moved to an intrafibre location inaccessible to the

K-microelectrode or that the free solvent water fraction (α^1 in equation 3 of Chapter V) increased at the expense of another internal compartment. However, the observed increase in $(Na)^1$ would argue against such an event, unless the free compartment, α^1, is relatively small. For simplicity, if one assumes that α^1 does not change and that the uptake of Na is confined to α^1 then $\alpha^1\Delta(Na)^1 = \Delta(Na)i$.. From the data of Table 1 the initial value α^1 cannot exceed 34% of the total intrafibre volume if a shift of intrafibre water is to account for the observed fall in $(K)^1$.

8.2.3 CHANGES IN THE FREE AND TOTAL K AND Na DURING A 40 MINUTE EXPOSURE TO NORMAL RINGER CONTAINING OUABAIN

According to our current knowledge of the Na pump, K-free Ringer and ouabain both block the normal function of the Na pump, consequently, one expects that their effects on intrafibre electrolyte distribution should be similar. But, since Corrie and Bonting (1966) had observed a definite and significant loss of total K after 4 hours of ouabain treatment whereas Simon et al. (1957) did not detect much loss after 4 hours in K-free Ringer, it seemed important to establish once and for all whether the transmembrane and intrafibre movements of K are indeed different for the two treatments.

The experimental protocol was identical to that of the previous section except that the superfusate was changed from normal Ringer to normal Ringer containing 10^{-4} M oua-

bain instead of to K-free Ringer. The results are summarized in Table 4 and for brevity only the mean changes are listed as in the bottom row of Table 3. For convenience and clarity, Table 5 lists the mean changes from Tables 3 and 4.

As with K-free Ringer treatment, there was a significant fall in $(K)^1$ (-27mM), and a small but significant increase in $(Na)^1$ (+5mM). Unlike the K-free treatment, however, there was a significant fall in (K)i of 12m.moles/kg fibre water. The increase in (Na)i of 7m.moles/kg fibre water was similar to that of K-free treatment. The most important point to be derived from these results in comparison with the data from the K-free exposure (see Table 5) is that while both treatments block the Na pump as shown by the increases in $(Na)^1$ and (Na)i, their effects on the intrafibre ionic distribution differed markedly. That is, both caused a decrease in $(K)^1$, but only ouabain initiated a transmembrane efflux of K. It should be pointed out that neither procedure caused an observable change in the muscle water content.

The data of Table 3 also points out that the loss of (K)i during ouabain clearly demonstrates that if the fall in $(K)^1$ observed during K-free exposure did actually cross the sarcolemma it should have been detected by chemical analysis. Therefore, the discrepancy between $(K)^1$ and (K)i during K-free exposure was not caused by delays in the extracellular space. A more theoretical treatment of this problem is presented in Appendix B.

Table 4: CHANGES IN FREE AND TOTAL K AND Na DURING A 40 MINUTE SUPERFUSION WITH 10^{-4} M OUABAIN.

	FREE CONCENTRATION		TOTAL CONCENTRATION	
Em(mV)	$(K)^1$	$(Na)^1$	(K)i	(Na)i
	(mM)		(m.moles/kg f.w.)	
0±0.8 (42)	-27±5.0 (20) p<0.001	+5±0.5 (22) p<0.001	-12±1.8 (36) p<0.001	+7±1.1 (36) p<0.001

All values are the changes during a 40 minute exposure to normal Ringer containing 10^{-4} M ouabain.

The p values are the probability that the difference is zero. The values in brackets are the degree of freedom.

From these arguments, it can now be concluded with greater conviction that K-free Ringer and ouabain have very different effects on the monovalent cation distribution in frog skeletal muscle and it clearly shows that K-free Ringer can induce an acute change in $(K)^1$, the free K, without a concomitant change in (K)i, the total intrafibre K. The fact that there was a loss in $(K)^1$ and (K)i during ouabain treatment shows that the disappearing free K was caused by an efflux of K rather than an increase in myoplasmic solvent thus justifying the initial assumption of Chapter (V).

Table 5: COMPARISON OF THE CHANGES IN FREE AND TOTAL K AND Na MADE BY K-FREE RINGER AND BY NORMAL RINGER CONTAINING 10^{-4}M OUABAIN.

		FREE CONCENTRATION		TOTAL CONCENTRATION	
TREATMENT	Em(mV)	$(K)^1$	$(Na)^1$	$(K)i$	$(Na)i$
		(mM)		(m.moles/kg f.w.)	
K-free Ringer	-18±1.6 (38) p<0.001	-36±1.8 (22) p<0.001	+16±1.7 (14) p<0.001	-1±2.7 (38)	+5.5±1.6 (18) p<0.002
Ouabain	0±0.8 (42)	-27±5.0 (20) p<0.001	+5±0.5 (22) p<0.001	-12±1.8 (36) p<0.001	+7±1.1 (36) p<0.001

Moreover, the fact that $\Delta(K)^1 > \Delta(K)i$ (-27mM vs -12m.moles/kg fibre water), is evidence that the free compartment cannot contain all of the fibre water and it follows that the fibre must contain at least one other compartment with or without K.

A simple first approximation model for the distribution of ions was developed in Chapter V. Substitution of the values of Table 4 into equations 8 and 9 of Chapter (V) gives a value of 0.23 for α^1 and 0.77 for α^2 which suggests that the free ions (as measured with the ion-selective microelectrodes) are dissolved only in 23% of the intrafibre

water and that the remaining 77% is to be found in an organelle (or organelles) not sampled by the microelectrodes.

If these α values are to be accepted they must be shown to have morphological significance. An examination of the stereological work of Mobley and Eisenberg (1975) reveals that such a large α^2 (77%) can only be related to the myofibrils, an organelle which occupies 83% of the intrafibre volume. If so, then the α^1 water must represent the extramyofilrillar space which is indeed where the tips of the ion-selective microelectrodes are usually located. The sarcoplasmic reticulum can be rejected as a compartment since Somlyo et al. (1977a,b and 1981) have reported that the sarcoplasmic reticulum contains a level of K and Na essentially identical to that of the sarcoplasm.

Implicit in this model, although not stated, is the supposition that an equilibrium boundary or interphase must exist between the two intrafibre compartments. Within the context of the muscle fibre morphology, there is an interphase around each myofibril. But, the myofibrils though well defined morphologically do not possess a limiting membrane. However, they do have their own unique Donnan potential (Weiss et al., 1967; Collins and Edwards, 1971; Pemrick and Edwards, 1974; Bartels and Elliott, 1980; Hinke, 1980; and Stephenson et al., 1981) because of the net negative charges on the myofilaments (both thick and thin) at physiological pH. It should be emphasized here that a membrane is

not essential for the existence of a potential between two phases. What is essential though, is that there must be a concentration gradient of the diffusible ions between the two phases effected by, for example, a membrane or by fixed charges such as those on the myofilaments.

Since no complete membrane envelopes the myofibrils (the S.R. forming only a discontinuous envelope) it follows that ions should move freely between the myoplasm and the myofibrils but probably in a way that only permits electroneutral exchanges to occur between the two compartments. This can be done by an exchange of co-ions or by a concomitant movement of counterions.

For the ouabain treatment, a 1:1 exchange of myofibrillar K for myoplasmic Na has already been assumed in order to calculate α^1 and α^2. With this assumption and application of the Table 4 data to equations (6) and (7) of Chapter (V) it was calculated that 7.5mM of myofibrillar K exchanged for a similar amount of myoplasmic Na during the 40 minute treatment. Figure 1 in Chapter V summarizes schematically the transmembrane and internal K:Na exchanges that have occurred during the 40 minute treatment with ouabain.

8.2.4 RE-EVALUATION OF THE K-FREE RINGER INDUCED CHANGES

In the K-free Ringer superfusion experiments, if equations (6) and (7) (Chapter (V)) are applied to the data of Table 3 and if the newly calculated values of α^1 and α^2 (from the ouabain data) are used, then one calculates that

the myofibrillar concentrations of K and Na increased by 10mM and 2mM respectively while myoplasmic K decreased by 36mM and myoplasmic Na increased by 16mM. Clearly, a 1:1 exchange of myofibrillar Na for free K cannot be used to explain the data of Table 1. If electroneutrality is to be maintained in α^2 and if the net fixed charges remained constant, then one must either postulate that an unknown cation was displaced from the myofibrils when K and Na entered or permit an anion to accompany the inward movment of K and Na. This latter hypothesis is less likely since the myofibrils are known to exclude anions (Hinke, 1980; Stephenson et al., 1981). Hence, I prefer the first hypothesis where an unknown cation, X, is in some way encouraged to leave the myofibrils for the myoplasm. The ionic movements during a 40-minute treatment with K-free Ringer is summarized schematically in Figure 8.

However, as discussed previously, an increase in myoplasmic solvent can also account for the simultaneous increase of free Na, and the decrease of free K, if the myoplasmic fraction of water was small and if the increase of total intrafibre Na concentration, was confined to the myoplasmic space. Since α^1 was calculated to be 0.23 thus it seemed prudent to re-consider this hypothesis. The intracellular changes of this hypothesis are schematically represented in Figure 9. According to this scheme the increases in myoplasmic water must be from α^2 (i.e. an intrafibre

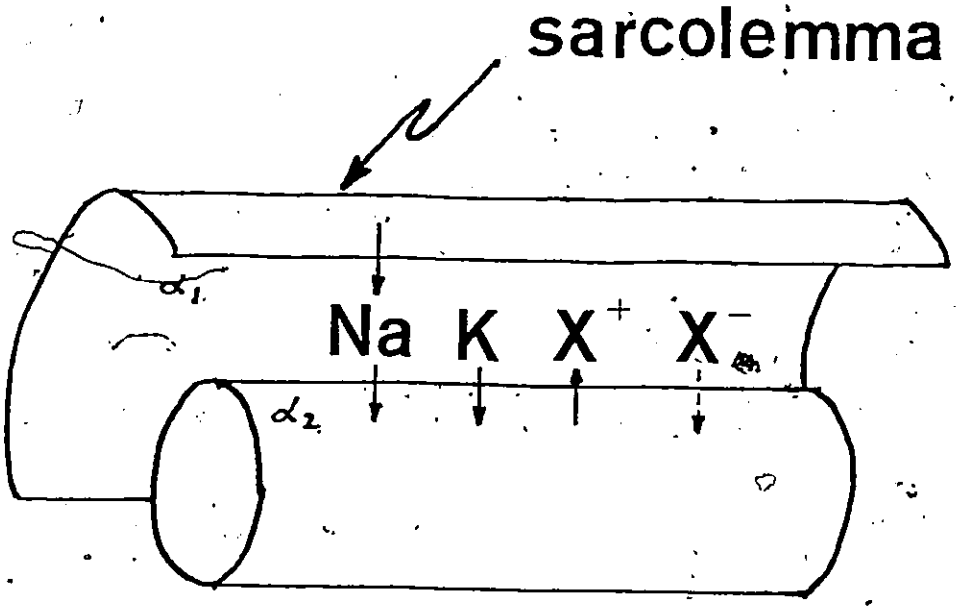

Figure 8: Schematic representation of the ionic shift model according to the two compartment model during a 40 minute exposure to K-free Ringer. There is an influx of Na across the sarcolemma and an internal sequestration of free K and to a lesser extent an internal sequestration of free Na by the myofibrils. The internal sequestration of K and Na is, for reasons of electroneutrality (see text), an exchange for a cation in the myofibrils (solid arrow) or a concomitant influx of an anion (dashed arrow).

redistribution of water), since there was no detectable change in the total muscle water. Since (K)i remained constant then one can write

$$\alpha^1_{40}(K)^1_{40} = \alpha^1_0(K)^1_0 \qquad (1)$$

where the subscripts denote the time in K-free Ringer. Using the data of Table 3, the calculated value of α^1_{40} is 0.32 which suggests that α^1 increased from 23 to 32% of the intrafibre water. The crucial test for this model is its prediction of α^1 from the Na data (Table 3). As before, it was assumed that the increase of (Na)i is confined to α^1, thus one can write

$$\alpha^1_{40}(Na)^1_{40} = \alpha^1_0(Na)^1 + \Delta(Na)i \qquad (2)$$

From this relation, the calculated α^1_{40} is 0.29, very close to that calculated from the K data (0.32). Thus it appears that either model can account equally well for the observed changes during a 40 minute exposure to K-free Ringer. However, it is hard to imagine how an uptake of Na by the muscle can be confined to the myoplasmic water since it has already been argued that ions are freely mobile between α^1 and α^2. Therefore, for subsequent arguments, the ion-shift model of Figure 8 will be favoured over the water-shift model of Figure 9.

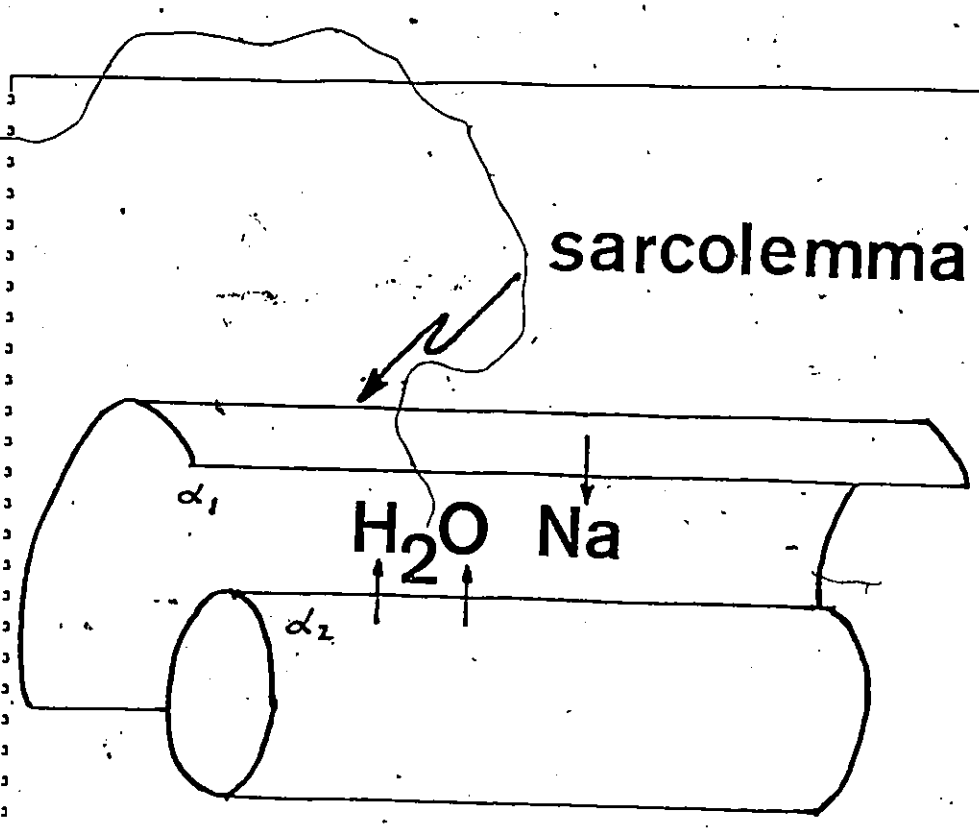

Figure 9: Schematic representation of the water shift model during a 40 minute exposure to K-free Ringer. There is an influx of Na across the sarcolemma which is confined to the myoplasm concurrent with a shift of water from the myofibrils to the myoplasm. The measured fall in free K is an effect of dilution of the free K in the myoplasm.

8.2.5 CHANGES IN $(K)^1$, AND (K)i AFTER AN 80 MINUTE EXPOSURE TO K-FREE RINGER vs CHANGES AFTER A 40 MINUTE EXPOSURE TO K-FREE RINGER

When a frog skeletal muscle is exposed to K-free Ringer for extended periods at low temperatures there is a loss of total intrafibre K and a gain in intrafibre Na (Steinbach, 1940; Kernan, 1962; Adrian and Slayman, 1966; Armstrong and Lee, 1971; and Kernan and MacDermott, 1975). In the acute exposures to K-free Ringer reported here there was a large fall of free K yet total intrafibre K remained constant, therefore, the acute effects must be a temporary phenomenon and intrafibre K must eventually leave the muscle fibre. To obtain supporting evidence for this idea, a group of preliminary experiments were performed which only compared the effects of a 80 minute treatment with K-free Ringer with the effects of a 40 minute treatment with K-free Ringer.

Paired muscles were used. $(aK)^1$ and Em were measured in several surface fibres in normal Ringer and in K-free Ringer in both muscles. In one muscle, the exposure to K-free Ringer was for 40 minutes while its companion muscle was exposed to K-free Ringer for 80 minutes. For chemical analysis of (K)i, two additional pairs were used. In one pair one muscle was exposed to K-free Ringer for 40 minutes while its companion was stored in normal Ringer. In the second pair, one muscle was exposed to K-free Ringer for 80 minutes while its companion muscle was stored in normal Ringer. The changes in $(K)^1$, and (K)i and Em are summarized in Table 6.

Table 6: CHANGES IN FREE AND TOTAL K DURING A 40 MINUTE AND AN 80 MINUTE EXPOSURE TO K-FREE RINGER.

TIME IN K-FREE RINGER	Em(mV)	$(K)^1$(mM)	(K)i (m.moles/ kg f.w.)
40 minutes	-18±0.9 (18) p<0.001	-35±1.0 (18) p<0.001	+1.5±1.5 (26)
80 minutes	-21±0.8 (18) p<0.001	-37±2.0 (18) p<0.001	-5±2.0 (22) p<0.025

The 40 minute exposure data confirms the main result of Table 1, that is, a large fall in $(K)^1$ while (K)i remained constant. The 80 minute data shows once again a large fall in $(K)^1$ but now there is a small but significant fall in (K)iindicating that some loss of total intrafibre K can be detected after 80 minutes but not after 40 minutes of exposure to K-free Ringer. As before there were no detectable changes in the muscle water content for either the 40 or 80 minute exposures. Lee and Armstrong (1974) during a 48 hour exposure to K-free Ringer observed a 20mM fall in their total intrafibre K but observed only a 14mM fall in their free K - the relative magnitude of the changes in the free and in the total K were opposite those observed in this thesis. It seems logical to speculate that during the period

from 80 minutes to 48 hours as more fibre K leaves the muscle fibre that the K sequestered by the myofibrils will be released back into the myoplasm.

8.2.6 REVERSAL OF THE 40 MINUTE EXPOSURE TO K-FREE RINGER

A frog skeletal muscle exposed to K-free Ringer (Adrian and Slayman, 1966; Steinbach, 1940) or to low K (0.2mM) Ringer (Johnson, 1956; and Desmedt, 1953) for extended periods re-accumulates its lost K and extrudes its gained Na when returned to K containing Ringer. The long term effects of K-free Ringer are reversible. Therefore, it was elected to examine the reversal of the 40 minute exposure to K-free Ringer.

Paired muscles were used. In one muscle, $(aK)^1$ or $(aNa)^1$ and Em were recorded in normal Ringer and after 40 minutes in K-free Ringer. $(aK)^1$ or $(aNa)^1$ and Em were recorded in its companion muscle after 40 minutes in K-free Ringer and after a 40 minute return to normal Ringer. For chemical analysis paired muscles were dissected and seperated into two groups of matched pairs. In one group, one muscle of each pair was transferred to K-free Ringer for 40 minutes while its companion muscle remained in normal Ringer. At the end of this period the muscles were all exposed to K-free Ringer for 40 minutes. One muscle of each pair was removed for analysis of K, Na and water content. Its companion muscle was returned to normal Ringer for 40 minutes then removed for analysis of (K)i, (Na)i and water content. The results of the averaged changes are summarized in Table

7. There were no detectable changes in the muscle water content during any of the experimental steps.

The data from the 40 minute exposure to K-free Ringer were consistent with similar experiments (Tables 3 and 6). That is, a fall in $(K)^1$ while (K)i remained constant and an increase of $(Na)^1$ greater than the increase of (Na)i. The recovery data, however, was unexpected : $(K)^1$ increased as expected but $(Na)^1$ did not decrease as expected. Furthermore, K now left the fibre and Na entered the fibre. These changes are opposite to the transmembrane ionic fluxes expected when the Na pump is re-activated by the re-addition of external K and perhaps at an accelerated rate due to the higher $(Na)^1$ level. It appears that short term exposures to K-free Ringer produce irreversible changes in the electrolyte distribution of frog skeletal muscle that have not been previously detected. Indeed, Desmedt (1953) chose 0.2mM K Ringer over K-free Ringer to alter the electrolytes in his experiments because K-free Ringer produced some unspecified irreversible changes.

The ionic shifts during recovery are schematically represented in Figure 10. Applying equations (6) and (7) of Chapter (V) to the data of Table 7 gives values of $(K)^2$ and $(Na)^2$ of -13mM and 9mM respectively. The scheme of Figure 10 indicates that the unkown cation lost during the K-free Ringer treatment did not completely return to ^{2} upon the re-introduction of normal Ringer (see Chapter IX). Perhaps

Table 7: CHANGES IN FREE AND TOTAL K AND Na DURING A 40 MINUTE RECOVERY FROM K-FREE RINGER.

Superfusate	Em(mV)	$(K)^1$	$(Na)^1$	(K)i	(Na)i
		(mM)		(m.moles/kg f.w.)	
40 minutes K-free Ringer	-16±1.1 (24) p<0.001	-35±2.0 (12) p<0.001	+15±1.0 (12) p<0.001	0±1.5 (24)	+5±1.0 (24) p<0.001
40 minutes recovery[3]	+15±1.0 (22) p<0.001	+21±3.0 (12) p<0.001	0±1.6 (10)	-5±2.5 (24) p<0.05	+7±0.8 (24) p<0.001

[3] Changes in this row are referred to measurements after a 40 minutes exposure to K-free Ringer and not to normal Ringer at the start of the experiment.

the altered distribution of the unknown cation is the key to the unexpected pattern of recovery.

The data of Table 7 is incompatible with the water redistribution model, if one postulates that α^1 must revert back to 0.23 from 0.32. That is, $(K)^1$ should increase and does so accordingly, while $(Na)^1$ should increase markedly since (Na)i was observed to increase. The fact that $(Na)^1$ remained constant is further evidence that the water redistribution model can be abandoned.

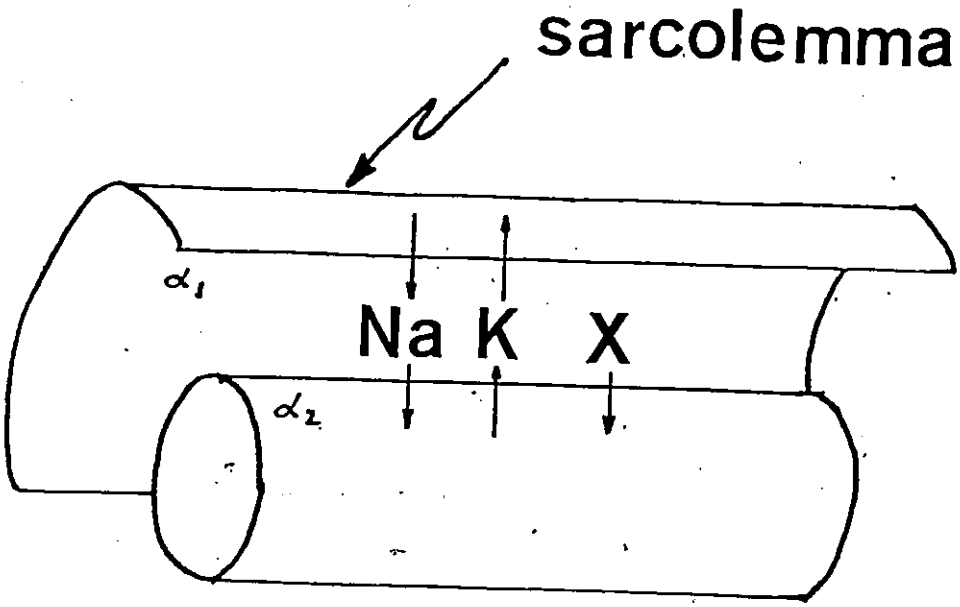

Figure 10: The ionic movements during a 40 minute recovery from K-free Ringer. There is an internal uptake of Na and an internal loss of K.

According to the two compartment model the X cation lost during the initial exposure to K-free Ringer does not return completely to the myofibrils. Notice that the transmembrane fluxes of K and Na are opposite those expected for a re-activated Na/K pump.

8.2.7 INDEPENDENT MECHANISMS OF K-FREE RINGER AND OUABAIN IN PERTURBING THE INTRAFIBRE MONOVALENT CATION DISTRIBUTION

Within the framework of the ion-shift model, K-free Ringer produces an internal exchange of myoplasmic K and Na for an unknown cation in the myofibrils (Figure 8) whereas ouabain produces an internal exchange of myoplasmic Na for myofibrillar K in addition to transsarcolemmal fluxes of K and Na (Figure 1, Chapter V). This set of experiments examines the interaction of the two treatments on the intrafibre K distribution.

Paired muscles were used. In one muscle, $(aK)^i$ and Em were measured in normal Ringer then after 40 minutes in K-free Ringer. In its companion muscle, $(aK)^i$ and Em were measured in normal Ringer and after 40 minutes in K-free Ringer containing 10^{-4}M ouabain. For chemical analysis, paired muscles were separated into two groups. In one group, one muscle of the pair was exposed to K-free Ringer for 40 minutes while its companion remained in normal Ringer. The muscles were analyzed for K, Na, and water content. The second group was treated in an identical manner as the first group except that one muscle of the pair was exposed to K-free Ringer containing 10^{-4} M ouabain. The averaged changes are summarized in Table 8. As before no change in the muscle water content was detected.

Table 8: CHANGES IN FREE K AND TOTAL K AND Na DURING A 40 MINUTE EXPOSURE TO K-FREE RINGER AND TO K-FREE RINGER CONTAINING OUABAIN

Superfusate	Em (mV)	$(K)^1$ (mM)	(K)i (m.moles/kg f.w.)	(Na)i (m.moles/kg f.w.)
K-free Ringer	-12±1.0 (16) p<0.001	-58±7.0 (16) p<0.001	0±2.0 (6)	+5.6±1.5 (6) p<0.025
K-free Ringer $+10^{-4}$M Ouabain	-11±1.0 (14) p<0.001	-58±7.0 (14) p<0.001	-9±2.0 (6) p<0.01	+8±1.5 (6) p<0.005

The K-free Ringer data (upper row) confirm the data of similar experiments (Tables 3,6,and 7), although the fall in $(K)^1$ was larger. The K-free Ringer plus ouabain data (lower row) revealed an equivalent fall in $(K)^1$ but in addition a significant fall in (K)i of 9m.moles/kg fibre water. This result demonstrates that transmembrane K movements are accelerated by ouabain but that the K-free Ringer induced internal K shift might still be present. Applying equation (5) of Chapter (V) to the data of Table 8 reveals that $(K)^2$ increased by about 5mM in K-free Ringer containing 10^{-4} M ouabain compared with about 16mM in K-free Ringer. Evidently, the internal redistribution of K was still present albeit attenuated. Thus during exposure to K-free Ringer

with ouabain both mechanisms of pertufbations of the muscle's normal electrolyte distribution were present but antagonistic with respect to $(K)^2$. It follows that K-free Ringer and ouabain have independent machanisms, both capable of lowering free K but only ouabain produces an acute loss of total intrafibre K.

8.3 DISCUSSION

The most important conclusions from these experiments and analyses are: (i) that free and total intrafibre K and Na concentrations are quantitatively different; (ii) that intrafibre K and Na must exist in two different concentrations in at least two distinct compartments; (iii) that the two compartments are probably the myofibrils and the extramyofibrillar myoplasm; (iv) that ions move freely between the two compartments subject only to the rule of electroneutrality; and (v) that such internal ionic movements do indeed occur in addition to transmembrane ionic movements; and (vi) that K-free Ringer treatment and ouabain treatment produce quite different internal ionic and transmembrane ionic shifts.

The key observation was that K-free Ringer treatment produced a large drop in free intrafibre K, detectable in 40 minutes without producing a similar reduction in the total intrafibre K. This observation alone strongly suggests that an internal redistribution of intrafibre K had occurred during the K-free Ringer treatment.

It is somewhat surprising that such an important observation has remained undetected for so long. Perhaps the answer lies in the fact that few past investigators had combined chemical analysis with ion-selective microelectrode recordings in experiments designed to invoke large transmembrane ionic exchanges. For example, Adrian (1956) and Simon et al. (1957) studied the effects of K-free Ringer on intrafibre K levels but were resticted to chemical analysis, hence had no way of detecting the intrafibre redistribution of K. Others have done microelectrode recordings and chemical analysis but have been concerned mainly with the normal condition (for example, Lev, 1964; and Kostyuk et al., 1969). Notable exceptions are Armstrong and Lee (1971) and Lee and Armstrong (1974). Their experiments were very similar to ones reported here. Unfortunately, they were interested only in examining the effects of 24 and 48 hour exposures to cold K-free Ringer and not in the early induced ionic changes. Nevertheless, their study permitted them to arrive at conclusions (i), (ii) and (v). Among the secondary observations, perhaps the most interesting was the failure of the K-free Ringer induced changes to reverse themselves when the muscle was returned to normal Ringer. Even more puzzling was the transmembrane exchange of intrafibre K for external Na which was exactly opposite to that expected if the Na/K pump had been re-activated. To explain this observation, it was proposed that the unknown cation

released during the initial K-free Ringer exposure might have become preferentially bound, hence was unable to return to its original myofibrillar location during the subsequent recovery. Alternatively, the unknown cation might have altered the normal forward mode of the Na/K pumpsuch that the pump now operates in reverse. Another possibility is that normal Ringer did not contain sufficient K to reverse the redistributed ions since previous workers have used Ringer containing 10 mM K as their solution following Na-enrichment (Adrian and Slayman, 1966; Kernan, 1962; Johnson, 1956; Desmedt, 1953; and Steinbach, 1940). This, however, is unlikely since Frumento (1965) found that Na-enriched muscles could extrude Na when returned to normal Ringer (2.5mM K).

A number of practical considerations arise from these experiments. Many workers have used isotopes, chemical analysis or ion-selective electrodes alone to study transport rates across the sarcolemma. But since the muscle fibre is non-homogeneous and since intrafibre exchanges occur in addition to the sarcolemmal exchanges any one of these techniques used alone cannot provide a true picture of all intrafibre events especially when the steady state of the muscle is perturbed. Since most of our knowledge on ion transport has come almost exclusively from isotope flux studies, perhaps one should critically re-examine data of this type in order to pinpoint possible errors which might arise when internal ionic shifts occur.

It is also worth mentioning that many properties of the plasma membrane are dependent on transmembrane ionic gradients which, this thesis reveals, should not be derived from chemical analysis. Strictly speaking, ionic activities should be used. For example, Kernan (1962), Frumento (1965) and Adrian and Slayman (1966) measured Em under conditions when the Na pump was electrogenic and deduced the contribution of the electrogenic Na pump to the membrane potential as the difference between the measured membrane potential and the K equilibrium potential, Ek, calculated from the K gradient across the membrane determined by chemical analysis. The use of activities rather than concentrations would not change the conclusion that a highly active Na pump can be electrogenic. Nevertheless, ionic activities are needed to define precisely the relation between Em and Ek and the precise electrogenic component of the Na pump.

Activity measurements alone cannot determine how the various fractions of intrafibre ions are affected by a highly active Na pump. Another shortcoming of relying on activity measurements alone can be illustrated by the data of Table 4. Ouabain must have induced an increase in the permeability ratio of K to Na, P_K/P_{Na}, at the sarcolemma, since Em remained constant while $(K)^1$ decreased and $(Na)^1$ increased. Unless permeability measurements are made one cannot concluded the precise cause of the increase in P_K/P_{Na}. The only safe conclusion that can be made is that

an increase of P_K/P_{Na} had ocurred. This observation has already been suggested by Sjodin and Beauge (1973).

8.3.1 CALCULATION OF α^1 AND α^2

In Tables 3 and 4, the observation that $\Delta(K)^1 \neq \Delta(K)i$ and $\Delta(Na)^1 \neq \Delta(Na)i$, are consistent with the existence of an intrafibre compartment not measured by the ion-selective microelectrode but involved in K and Na exchange. If the fibre interior was indeed homogeneous then $\Delta(K)^1 = \Delta(K)i$ and $\Delta(Na)^1 = \Delta(Na)i$. This conclusion corroborates that of Armstrong and Lee (1971) and Lee and Armstrong (1974) who performed similar experiments. It was shown in Chapter II that the heterogeneous distribution of monovalent cations is caused by the macromolecules of the contractile filaments. This binding of ions should give rise to an intrafibre Donnan potential.

As a point of interest the intrafibre Donnan potential was calculated according to the relationship $(K)^2+(Na)^2/(K)^1+(Na)^1 = \exp(E_D F/RT)$ where $RT/F = 25.2mV$. Using the data of Table 1 and values of 0.23 and 0.77 for α^1 and α^2 respectively to calculate $(K)^2$ and $(Na)^2$ a -5mV intrafibre Donnan potential was obtained. This value is close to the measured value of -8mV in glycerol extracted frog sartorius muscles (Collins and Edwards, 1971) and also close to the measured value of -9mV in mechanically skinned toad iliofibularis muscles (Stephenson et al., 1981). However, it is unrealistic to expect ions in a muscle fibre to be

divided into two intrafibre compartments only given the complex morphology of a muscle fibre. For example, the sarcoplasmic reticulum, nuclei and mitochondria all occupy a finite volume and thus an exact correspondence between the calculated and the measured value cannot be expected. Nevertheless, a closer agreement between the calculated and measured values is possible if it is assumed that the non-solvent water is equivalent to the non-osmotic water (Hinke, 1970) and resides in α^2 [16] In the frog sartorius muscle about 15% of the intrafibre water is non-osmotic (see Chapter I) the α^1 and α^2 values are then 0.23 and 0.62 respectively. Applying these values to the data of Table 1 to once again calculate $(K)^2$ and $(Na)^2$ gives a -10mV intrafibre Donnan potential. However, for the sake of simplicity α^1 and α^2 will be assumed to be 0.23 and 0.77 respectively for future calulations. This simple calculation shows that it is reasonable to speculate that the myofibrillar accumulation of cations could well be the in vivo fraction of monovalent cations which is not detected by the ion-selective microelectrode.

Enough supporting evidence has been provided for the existence of an ionic Donnan distribution between the myofibrils and its surrounding medium. There remains, however, a question concerning the exact location of the Donnan potential generated by the seperation of cations and anions due

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[16] α^2 denotes all intrafibre water excluding the free compartment.

to the fixed negative charges on the contractile filaments. For example, Hinke (1980) argued that the true Donnan potential should be located inside the myofibril close to the surface of the contractile filament whereas Stephenson et al. (1981) argued that the true Donnan potential should be located at the myofibrillar surface. Measurements of the myofibrillar Donnan potential have been done by inserting an open-tipped microelectrode filled with KCl into the myofibril. Because of the size of the microelectrode tip in comparison with the size of the myofilament lattice there is some doubt whether the measured potential is a measure of the true potential at the surface of the contractile filaments.

Elliott and Bartels (1981) and Naylor (1981) have shown from a comprehensive theoretical study of the electrostatic potentials at the surfaces of the myofilaments and compared their magnitudes with the potentials actually measured by the open-tipped microelectrodes and which appear to relate to the whole myofibril rather than to the myofilaments. Their analyses lead to the conclusion that the measured myofibrillar Donnan potential [17] should not be too different from the calculated myofilament electrostatic potential, at least at physiological pH conditions. Thus, for practical

[17] Strictly speaking, the Donnan potential is the potential between two bulk phases with different concentrations of diffusible ions and therefore cannot account for the true electrostatic potential at the surface of the myofilaments. The interested reader is referred to an excellent and thorough review on the theory of the Donnan potential by Overbeek (1956).

purposes, it can be imagined that the measured Donnan potential is located at or near to the myofibrillar surface. It can be imagined that the freely mobile and exchangeable ions within the myofibrils are partitioned in a way as defined by the Donnan potential.

Truly site bound cations (or anions) to the myofilaments of course cannot contribute to the Donnan potential and need not obey the Donnan distribution. if a fraction of the K and Na in the myofibrils are truly site bound then the analyzed myofibrillar K and Na should be larger than predicted from the Donnan potential. But, Hinke (1980) found that the measured myofibrillar K and Na concentrations were close to that predicted by the measured Donnan potential. Therefore, as a first approximation, one may assume that the bulk of the intra-myofibrillar K and Na are relatively free and mobile, but functioning as counterions for the fixed negative charges on the myofilaments.

In deriving the two compartment model, no physico-chemical significance was assigned to the α^1 and α^2 water fractions until the physico-chemical significance of the cations associated with the water factions became obvious. Clearly, the α^1 water cannot be anything but free solvent water because the $(C)^1$ cations are defined as thermodynamically free (i.e. as measured by the ion-selective microelectrode) The α^2 water is , however, much more difficult to define, partly because it may contain more than one compart-

ment (although it has been argued that most of it must be myofibrillar), partly because some of the $(C)^2$ cations may well be truly site bound or at least restricted in the vicinity of fixed charge sites, and partly because the α^2 must also include all the intrafibre non-solvent water (because by definition α^1 contains none). If the results of Hinke (1970) can be used then 75% of the of the intrafibre water can act as solvent water for the monovalent cations, then a significant portion (i.e. 2/3) of the α^2 water should be reasonably normal water. The remaining portion (i.e. 1/3) of the α^2 water must, by definition, represent Hinke's non-solvent water which is structured around the macromolecules to some degree with altered physical properties (see Chapter I). It is not unreasonable to imagine that the myofibrils should include both structured and "normal" water when one considers that the myofibrils behave as a liquid crystal with both liquid and solid state properties.

8.4 SUMMARY

The key observation of this chapter is that there are intrafibre ionic movements when the normal ionic distribution of the frog skeletal muscle is perturbed. During the 40 minute treatment with K-free Ringer there was an internal sequestration of free K and to a lesser extent an internal sequestration of Na probably by the myofibrils in exchange for some unknown cation. During ouabain treatment there was

an internal exchange of free Na for myofibrillar K . These observations demonstrate clearly that K-free Ringer and ouabain do not have the same effect on intrafibre ionic distribution of monovalent cations despite a common attribute of Na/K pump inhibition. The analyses of the data suggest that it is the intrafibre compartmentalization of monovalent cations by the myofibrils that is responsible for the difference. Thus intrafibre compartmentalization plays a role in regulating the intrafibre distribution of monovalent cations.

Chapter IX

INTRAFIBRE CHANGES ASSOCIATED WITH K-FREE RINGER TREATMENT

9.1 INTRODUCTION

During K-free Ringer treatment it was postulated that the myofibrils sequestered 12 mEq/L of myoplasmic K and Na in exchange for an unknown cation. The possible candidates for the unknown cation are Mg, Ca and H. Of these, the most likely candidate is Mg because it is present inside the fibre at a reasonable concentration (ca. 15m.moles/kg fibre water) (Page et al., 1971; Brinley et al., 1977; and Lopez et al., 1983) and because much of it is known to be bound to the myofilaments (Brinely et al., 1974 and Naninga, 1961). It is, however, possible that instead of an efflux of a myofibrillar cation for the incoming K and Na, that the incoming K and Na might be accompanied by an anion. In order to rule out some of these possibilities, changes in intrafibre pH, free and total intrafibre Cl, and total intrafibre Mg and Ca were determined following K-free Ringer treatment.

9.2 RESULTS

9.2.1 FREE AND TOTAL INTRAFIBRE Cl IN NORMAL RINGER VS FREE AND TOTAL INTRAFIBRE Cl AFTER 40 MINUTES ON K-FREE RINGER

According to the two compartment model K-free Ringer treatment produces an intrafibre sequestration of K and Na by the myofibrils. This sequestration may be concomitant with a myofibrillar uptake of Cl to satisfy the condition of electroneutality. Frog skeletal muscles are highly permeable to Cl (Hodgkin and Horowicz, 1959; Hutter and Warner, 1967a, b, c, and 1972; and Eisenberg and Gage, 1969). Hence the Cl ion is accessible to the myofibrils from the extracellular space and could conceivably play a role despite a low initial intrafibre concentration (Kernan et al., 1974; and Bolton and Vaughan-Jones, 1976 and 1977). However, it is unlikely that the uptake of Cl plays a role in maintaining myofibrillar electroneutrality since Hinke (1980) has shown that the myofibrils exclude anions. Furthermore, the intrafibre Cl concentration is slaved to the membrane potential (Hodgkin and Horowicz, 1959). Nevertheless, it was elected to measure intrafibre Cl levels to ensure that nothing unusual had occurred.

Paired muscles were dissected and treated as described previously. One muscle was used for $(aCl)^i$ measurements in normal Ringer and after 40 minutes in K-free Ringer while its companion muscle remained in normal Ringer. Both muscle

were analyzes for total intrafibre Cl and water content. The results are summarized on Table 9.

As before, Em hyperpolarized and the muscle water content remained constant. Free intrafibre Cl, $(Cl)^1$, fell 2.4 mM which means that free Cl either moved into the myofibrils or moved out of the muscle fibre into the extracellular space as an adjustment to hyperpolarization. This latter possibility is supported by the total intrafibre Cl, (Cl)i, analysis which shows that (Cl)i remained constant.

Table 9: CHANGES IN FREE AND TOTAL Cl FOLLOWING A 40 MINUTE EXPOSURE TO K-FREE RINGER.

Superfusate	Em(mV)	$(Cl)^1$(mM)	(Cl)i (m.moles/kg f. w.)
Normal Ringer	-86±1.0 (9)	4.75±0.2 (4)	19.1±0.7 (20)
K-free Ringer	-101±0.6 (16)	2.32±0.2 (6)	18.1±0.6 (20)
Difference	-15±1.2 (16)	-2.4±0.28 (6)	-1±0.9 (38)

If myoplasmic Cl did indeed enter the myofibrils in sufficient quantities to maintain myofibrillar electroneutrality total intrafibre Cl would increase in detectable amounts.

Since (Cl)i remained constant, it seems appropriate to rule out any role for Cl in the K-free Ringer induced internal sequestration of free K.

9.2.2 INTRAFIBRE pH IN NORMAL RINGER VS INTRAFIBRE pH AFTER 40 MINUTES IN K-FREE RINGER

If the myofibrillar sequestration of K and Na displaced protons on the residues of the myofilaments then one must conclude that the K-free Ringer treatment had in some way dramatically altered the net negative charge on the myofilaments. Although this seems highly unlikely, nevertheless, it was decided to verify that intrafibre pH, pHi, remained constant during K-free Ringer treatment, as first observed by Kostyuk and Sorokina (1961).

Frog sartorius muscles were dissected and punctured in the usual manner with a pH microelectrode in normal Ringer and after 40 minutes in K-free Ringer. In five successful experiments pHi was 7.18±0.002(mean s.e., n=5) in normal Ringer and was 7.21±0.002 (mean s.e., n=5) after 40 minutes in K-free Ringer. It seems prudent then to discard the involvement of protons in the response to K-free Ringer.

9.2.3 TOTAL INTRAFIBRE Mg AND Ca CHANGES DURING A 40 MINUTE EXPOSURE TO K-FREE RINGER AND TO NORMAL RINGER CONTAINING OUABAIN

Ca and Mg are the remaining candidates. Ca can be ruled out partly because its intrafibre levels are too low and

partly because any release of Ca would elicit a contracture which was never observed. Thus Mg is left as the sole candididate.

Paired muscles were used. One muscle of each pair was stored in normal Ringer while its companion muscle was exposed to either K-free Ringer or to normal Ringer containing 10^{-4}M ouabain. Both muscles were analyzed for Ca and Mg as desribed in the Methodology section. The results are summarized in Table 10.

Clearly, no changes in the total intrafibre Ca or Mg had occurred during either K-free Ringer or ouabain treatment. From this, one can conclude that no significant loss of Mg or Ca had occurred. It is unlikely that Ca exchanged with K and Na for reasons already discussed. However, one cannot conclude that an internal Mg shift had not taken place. The role of Mg will have to await the arrival of a reliable means of measuring free Mg.

Table 10: TOTAL INTRAFIBRE Mg AND Ca FOLLOWING A 40 MINUTE EXPOSURE TO K-FREE RINGER AND TO NORMAL RINGER CONTAINING OUABAIN.

Superfusate	(m.moles/kg f.w.) (Mg)i	(Ca)i
Normal Ringer	14.0±0.5 (8)	3.3±0.2 (8)
K-free Ringer	14.0±0.5 (8)	3.3±0.2 (8)
Difference[5]	0±0.7 (14)	0±0.3 (14)
Normal Ringer	17.1±0.4 (8)	3.0±0.2 (8)
Normal Ringer + 10^{-4}M Ouabain	16.7±0.4 (8)	3.0±0.3 (8)
Difference[6]	-0.4±0.6 (14)	0±0.4 (14)

[5]Difference between matched pairs in normal Ringer and in K-free Ringer.

[6]Differnce between matched pairs in normal Ringer and in normal Ringer with 10^{-4} M ouabain.

9.3 DISCUSSION

The intrafibre distribution of Mg can be described by

$$(Mg)i = \alpha^1(Mg)^1 + \alpha^2(Mg)^2 + MgATP + MgR \qquad (1)$$

where (Mg)i, $\alpha^1(Mg)^1$, $\alpha^2(Mg)^2$ have significances analogous to the monovalent cations, MgATP is the concentration of MgATP and MgR is the concentration of residually bound Mg.

Lopez et al. (1983) found that the free Mg concentration in frog skeletal muscle was about .4mM. Brinley et al. (1977) found 4.2mM and 1 mM for MgATP and MgR respectively in barnacle muscle fibres. Applying these values to equation (1) gives a myofibrillar Mg concentration of 12mM. This calculation shows that the myofibrillar compartment does indeed hold enough Mg for its proposed role. The amount which would have to be displaced by K and Na is 6 mM. If 6 mM of myofibrillar Mg was indeed released into the myoplasm, the myoplasmic Mg concentration would increase 20Mm. This would satisfy the osmotic loss of 20 mM of monovalent cations by the myoplasm. However, it is hard to imagine how a simple deletion of external K could lead to an intrafibre sequestration of K and Na in exchange for Mg.

If K-free Ringer did indeed initiate an exchange of myofibrillar Mg for myoplasmic K and Na, one is still left with the task of finding a mechanism where a simple deletion of 2.5mM of K from the extrafibre space could elicit such a response. Perhaps, the removal of external K in some inexplicable way altered the charge density on the myofilaments such that K is preferred over Mg. It is known that myofilaments on barnacle muscle fibres alter their effective charge when induced to contract (Caille, 1981). Similarly, Bartels and Elliott (1981) have found that during rigor the A-band of skinned fibres undergoes a change in the Donnan potential, indicating that a the charge on the myofilaments had

occurred. The following two chapters will describe attempts to determine such a mechanism.

Chapter X

EXTERNAL SODIUM IS REQUIRED FOR THE DISAPPEARANCE OF FREE K IN RESPONSE TO K-FREE RINGER.

10.1 Introduction

This chapter reports on the effects of deleting external Na on the response of the muscle fiber to K-free Ringer. The rationale for making this variation is based on Henderson's finding (1971a) that K-free Ringer induced K movements could be modified by external Na. Ringer Na was replaced either by Tris or Li to safegaurd against the possibility that the substituting cation might be responsible for the results found. The experiments were carried out in the usual manner, except that both protocols were used, i.e. continuous recordings of free intrafibre K and before and after sampling of free intrafibre K and Na in single surface fibres.

10.2 RESULTS

10.2.1 EFFECT OF Na REMOVAL ON THE K-FREE RINGER INDUCED SEQUESTRATION OF FREE INTRAFIBRE K

Figure 11 is a summary of the continuous recording experiments. Notice that all muscles are first superfused for 30 minutes with K-and Na-free Ringer then half were superfused with K-free Ringer and the other half were superfused with normal Ringer for an additional 30 minutes. It is important to remember that both terminal superfusates contained normal Na.

Figure 11 clearly shows that $(aK)^1$ did not fall significantly when K-and Na-free Ringer was used but it did fall, rapidly and significantly, when the superfusate was switched to K-free Ringer. When the second solution was normal Ringer (with 2.5 mM K) there was no fall in $(aK)^1$. The upper curves show that hyperpolarization of the membrane potential occurred when external K was absent but disappeared when external K was restored. This experiment clearly shows that the K-free Ringer induced fall in free intrafibre K required the presence of external Na and was not dependent on hyperpolarization since it occurred during both K-and Na-free Ringer and K-free Ringer superfusions.

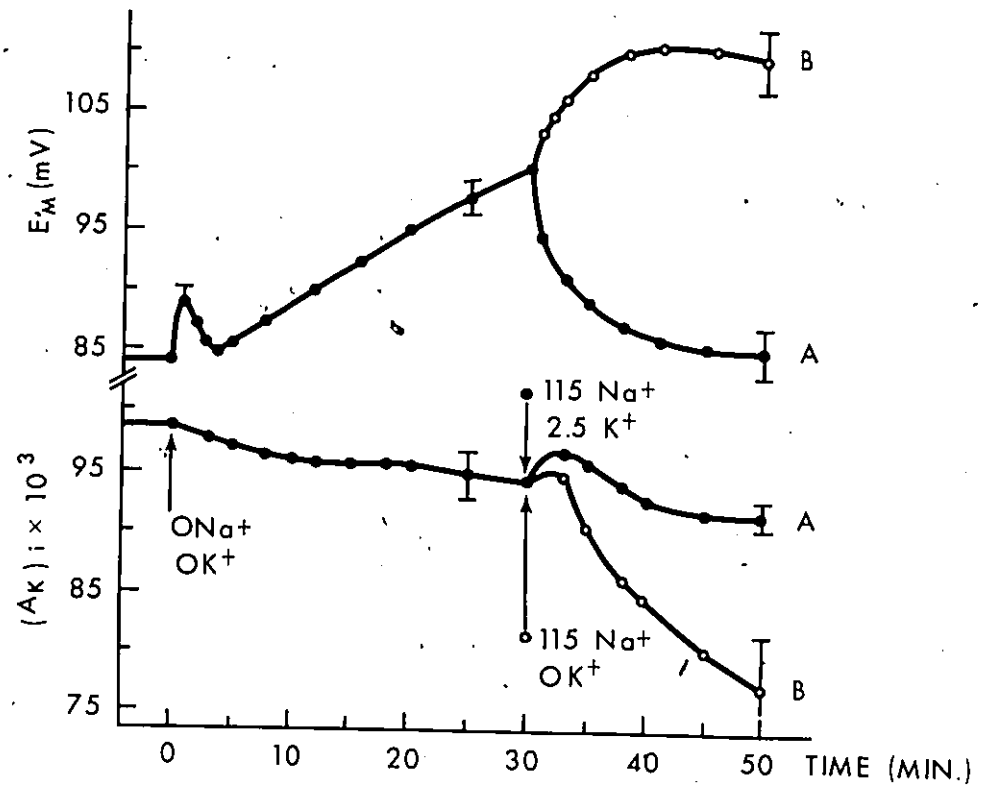

Figure 11: Averaged continuous recordings of $(aK)_i$ and Em when the superfusate is changed from normal Ringer to K-and Na-free Ringer (TRIS substituted) at t=o. At t=30 minutes the superfusate is changed to either K-free Ringer or back to normal Ringer. From t=0 to t=30 minutes, the curve is from 12 successful experiments and from t=30 to t=50 minutes each curve is from 6 successful experiments.

Table 11: CHANGES IN THE FREE AND TOTAL INTRAFIBRE K AND Na FOLLOWING A 40 MIN. EXPOSURE TO K-AND Na-FREE RINGER (TRIS SUBSITUTED).

Superfusate	Em(mV)	$(K)^1$	$(Na)^1$	(K)i	(Na)i
		(mM)		(m.moles/kg f.w.)	
Normal Ringer	-85±1.0 (27)	135±1.5 (17)	13±0.5 (10)	160±1.6 (15)	18±1.4 (15)
K-and Na-free Ringer	-101±1.2 (27)	130±2.5 (17)	7±0.5 (10)	159±2.1 (17)	9±0.5 (17)
Difference	-16±1.6 (52) p<0.001	-5±2.9 (32)	-6±0.7 (18) p<0.001	-1±2.6 (30)	-9±1.5 (30) p<0.001
Difference'	-18±1.6 (38)	-36±1.8 (22)	+16±1.7 (14)	-1±2.7 (38)	+5.5±1.6 (18)

'Difference is from Table 3 of Chapter VIII. Changes of free and total K and Na in K-free Ringer.

10.2.3 CHANGES IN FREE AND TOTAL INTRAFIBRE K AND Na DURING A 40 MINUTE EXPOSURE TO K-AND Na-FREE RINGER (Li SUBSTITUTED)

These experiments were identical to the previous group except Na was replaced by Li rather than by TRIS. The results are summarized in Table 12.

As before both $(K)^1$ and (K)i remained relatively unchanged while both $(Na)^1$ and (Na)i fell significantly.

Table 11: CHANGES IN THE FREE AND TOTAL INTRAFIBRE K AND Na FOLLOWING A 40 MIN. EXPOSURE TO K-AND Na-FREE RINGER (TRIS SUBSITUTED).

Superfusate	Em(mV)	$(K)^1$	$(Na)^1$	(K)i	(Na)i
		(mM)		(m.moles/kg f.w.)	
Normal Ringer	-85±1.0 (27)	135±1.5 (17)	13±0.5 (10)	160±1.6 (15)	18±1.4 (15)
K-and Na-free Ringer	-101±1.2 (27)	130±2.5 (17)	7±0.5 (10)	159±2.1 (17)	9±0.5 (17)
Difference	-16±1.6 (52) p<0.001	-5±2.9 (32)	-6±0.7 (18) p<0.001	-1±2.6 (30)	-9±1.5 (30) p<0.001
Difference'	-18±1.6 (38)	-36±1.8 (22)	+16±1.7 (14)	-1±2.7 (38)	+5.5±1.6 (18)

'Difference is from Table 3 of Chapter VIII. Changes of free and total K and Na in K-free Ringer.

10.2.3 CHANGES IN FREE AND TOTAL INTRAFIBRE K AND Na DURING A 40 MINUTE EXPOSURE TO K-AND Na-FREE RINGER (Li SUBSTITUTED)

These experiments were identical to the previous group except Na was replaced by Li rather than by TRIS. The results are summarized in Table 12.

As before both $(K)^1$ and (K)i remained relatively unchanged while both $(Na)^1$ and (Na)i fell significantly.

Since both TRIS and Li substitution produced the same effect it seeems appropriate to conclude that external Na is specifically required to initiate the internal sequestration of K by the myofibrils during K-free Ringer treatment. Perhaps it is the entry of Na which initiates the sequestration.

Table 12: CHANGES IN THE FREE AND TOTAL K AND Na FOLLOWING A 40 MIN. EXPOSURE TO K-AND Na-FREE RINGER (Li SUBSTITUTED).

Solution	Em(mV)	(K)^1	(Na)^1	(K)i	(Na)i	(Li)i
		(mM)		(m.moles/kg f.w.)		
Normal Ringer	-83± 0.8 (16)	143± 6.0 (11)	20± 1.9 (5)	134± 1.8 (18)	31± 1.7 (18)	
K-and Na-free Ringer	-104± 1.6 (18)	139± 1.8 (12)	7.3± 0.9 (6)	130± 2.0 (20)	11± 0.9 (20)	11± 1.5 (20)
Difference	-21± 1.8 (32) p<0.001	-4± 6.3 (21)	-13± 2.1 (9) p<0.001	-4± 2.7 (36)	-20± 1.9 (36) p<0.001	+11± 1.5 p<0.001

The differences between the mean initial values of free and total intrafibre K and Na in Tables 11 and 12 warrant comment. It was observed that total K and Na within the same group of frogs do not vary by more than ±6% but that considerably larger variations can be found between the muscles

from frogs obtained at different times of the year and from different sources. It was reported in Chapter (VII) that $(K)^1<(K)i$ in winter frogs and that $(K)^1>(K)i$ in summer frogs. Finally, the sum of (K)i and (Na)i may vary by as much as 20m.moles/kg fibre water in muscles from different frogs. But the response to changes in bath composition remains consistent as illustrated by comparing Tables 11 and 12.

10.3 DISCUSSION

The disappearance of free intrafibre K during K-free Ringer treatment required the presence of external Na because it fails to disappear either into the myofibrils or out of the muscle fibre when external Na is absent. The internal K-shift is apparently not dependent on hyperpolarization per se because the latter is observed to occur (Figure 11) without producing a fall in $(K)^1$.

Henderson (1971b) has suggested from his membrane potential masurements that Li is more permeable than TRIS in the frog sartorius muscle. This conclusion was based on the observation that K-and Na-free Ringer (TRIS substituted) produced an incremental hyperpolarization following an initial exposure to K-and Na-free Ringer (Li substituted). It is noteworthy that no additional hyperpolarization was seen here when TRIS was the Na substitute (compare the hyperpolarizations in Tables 11 and 12). However, the data of

Tables 11 and 12 were collected at different times of the year on frogs from different sources, hence a rigorous comparison cannot be made. Nevertheless, it is tempting to speculate that the larger fall in both $(Na)^1$ and (Na)i (Table 12 compared with Table 11) was caused by a greater influx of Li than TRIS which then displaces Na. Notice that in both TRIS and Li substituted Ringers the final values of $(Na)^1$ and (Na)i were very similar. Perhaps the larger fall in Na levels in the Li substituted Ringer may merely reflect the larger initial value of $(Na)^1$ and (Na)i since the fall of intrafibre Na is dependent on the gradient across the membrane (Vaughan-Jones, 1977) reaching an asymtotic value after about 30 minutes (White and Hinke, 1976 and Vaughan-Jones, 1977). The differences in the fall of intrafibre Na in the two experiments were essentially identical to the differences in the initial values. The data of Table 2 suggest that Li entered the muscle fibre and specifically displaced Na. This specific displacement of Na but not K by Li has been observed before in barnacle muscle fibre by Allen and Hinke (1971) and in the toad sartorius muscle by Simon et al. (1959). If Li did indeed enter the muscle fibre and specifically displaced bound Na but not bound K then the muscle fibre may possess at least two distinct sets of monovalent cation binding sites, one for Na one for K. Alternatively, there may be a rank order of monovalent cation binding (K>Li>Na). The latter hypothesis is unlikely as Hinke

et al. (1973) have demonstrated in barnacle muscle fibres that Na is bound preferentially over K in a ratio of about 3:1.

It should be pointed out that any interpretation of total intrafibre Na concentration must be approached with caution since a small error in the estimation of the extracellular space volume will result in a large error in the calculated total intrafibre Na concentration. Moreover, Ling and Walton (1975) have found that 9.1% of the wet weight of a frog (Rana pipien) is connective tissue. This connective tissue can bind up to 80 m.moles of Na/kg of connective tissue when equilibrated with normal Ringer. This is equivalent to 7.3 m.moles of bound Na/kg muscle wet weight. This estimate of extracellularly bound Na is in good agreement with a value of 8 mEq/kg muscle wet weight estimated by Carey and Conway (1957). This fraction of Na was considered to be extracellular since it was freely exchangeable. If Li did indeed displace externally bound Na then almost all of the observed Li uptake (Table 2) can be accounted for in the extracellular space. Thus, great caution must be exercised before one can conclude from chemical analysis alone that Li did actually enter the muscle fibre.

Finally, it is worth speculating on the fact that both K-and Na-free Ringer solutions produced very similar final values of of $(Na)^1$ and (Na)i. Perhaps this reflects the quantity of intrafibre bound Na. This thought will be pursued further in Chapter XII.

Regardless of the cause of the larger fall in intrafibre Na levels during Li substitution it is obvious that free intrafibre K does not disappear during K-free Ringer treatment unless external Na is present.

Chapter XI

DETUBULATION ABOLISHES THE RESPONSE TO K-FREE RINGER TREATMENT

11.1 INTRODUCTION

This group of experiments involved a re-examination of the intrafibre electrolyte response to K-free Ringer treatment in muscles which have been detubulated by the hypertonic glycerol shock method. The rationale for doing these experiments was based on the speculation that the transverse tubular system (tts) may the operative communicating pathway between the extracellular space and the myofibrils; and, if severed, the extracellular K-free condition might not be detected by the myofibrils. It is certainly well established that the tts communicates freely with the extracellular space(Huxley, 1964; Hill, 1964; and Endo, 1966) and that the tts transmits the electrical activity of the sarcolemma to the sarcoplasmic reticulum (Gage and Eisenberg, 1967; and Chandler et al., 1976). There is also evidence that the ionic transport properties of the tts membrane are different from those of the sarcolemma (Eisenberg and Gage, 1969; Lau et al., 1979 and Kovacs et al., 1981). It seemed plausible that the tts may also communicate extracellular electrolyte changes to the myofibrils since all myofibrils are close to

glutaraldehyde overnight then washed with 0.1M phosphate buffer (pH 7.6) containing 5% sucrose. These muscles were then embedded in 7% agar and cut to 65um thick sections with a Sorvol TC-2 tissue sectioner. The sections were then washed for 30 minutes in 0.1M phosphate buffer, then transferred to 0.05M TRIS-HCl buffer (pH 7.4) containing 0.05% diaminobenzidine (DAB) and 0.01% H_2O_2 and incubated for 30 minutes. The sections were then washed three times breifly in distilled water, then post-fixed with 1% OsO_4 in 0.1M phospate buffer for 2 hours. Dehydration of the sections was achieved by successive washes in alchohol increasing from 50 to 100% then with 100% propylene oxide. The dehydrated sections were then treated with a 1:1 solution of propylene oxide and an embedding media consisting Epon 812, Araldite 502 and dodecenyl succinic anhydride (DDSA) in a 1:1.25:1:3 ratio with tri(dimethyl amino methyl) phenol (DMP-30) overnight. The sections were then treated with embedding media alone and cured at 75 °C for 48 hours. The embedded sections were then cut on a Reichert OMU2 ultra microtome and viewed with a Philips 300 electron microscope.

The electrical continuity of the tts with the extracellular space was examined on paired whole muscles by extracellular stimulation through a pair of platinum electrodes in the recording chamber (Figure 6, Chapter VI). One muscle of each pair was exposed to the glycerol shock. The contraction was observed through the dissecting microscope in Fig-

glutaraldehyde overnight then washed with 0.1M phosphate buffer (pH 7.6) containing 5% sucrose. These muscles were then embedded in 7% agar and cut to 65um thick sections with a Sorvol TC-2 tissue sectioner. The sections were then washed for 30 minutes in 0.1M phosphate buffer, then transferred to 0.05M TRIS-HCl buffer (pH 7.4) containing 0.05% diaminobenzidine (DAB) and 0.01% H_2O_2 and incubated for 30 minutes. The sections were then washed three times breifly in distilled water, then post-fixed with 1% OsO_4 in 0.1M phospate buffer for 2 hours. Dehydration of the sections was achieved by successive washes in alchohol increasing from 50 to 100% then with 100% propylene oxide. The dehydrated sections were then treated with a 1:1 solution of propylene oxide and an embedding media consisting Epon 812, Araldite 502 and dodecenyl succinic anhydride (DDSA) in a 1:1.25:1:3 ratio with tri(dimethyl amino methyl) phenol (DMP-30) overnight. The sections were then treated with embedding media alone and cured at 75 °C for 48 hours. The embedded sections were then cut on a Reichert OMU2 ultra microtome and viewed with a Philips 300 electron microscope.

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ure 4, Chapter VI. The stimuli were single square wave pulses of varying amplitude and duration generated by a WPI stimulator model 301T. An excitability curve was generated for both the control and the glycerol shocked muscle.

The efficacy of the detubulation procedure and the damage sustained during the detubulation process were examined by exposing muscles to ^{14}C-inulin at various stages of the detubulation process. The inulin was filtered prior to use as described in Chapter (VI). The muscles were analyzed for ^{14}C-inulin content as described in Chapter (VI).

11.3 RESULTS

11.3.1 VERIFICATION OF DETUBULATION BY HORSE RADISH PEROXIDASE LOCALIZATION AND BY UNCOUPLING OF EXITATION AND CONTRACTION

Figure 12 was taken from a normal muscle in normal Ringer with HRP. The HRP reaction product is visible throughout both the extracellular space and the tts demonstrating that the tts in an untreated muscle communicates freely with the extracellular space. Figure 13A was taken from a glycerol treated muscle which was not exposed to HRP until 1 hour after the muscle was returned to normal Ringer. The HRP reaction product once again is seen in the extracellular space but no longer in the tts.

Figure 13B was taken from a glycerol treated muscle which was exposed to HRP 4 hours after the return to normal Ring-

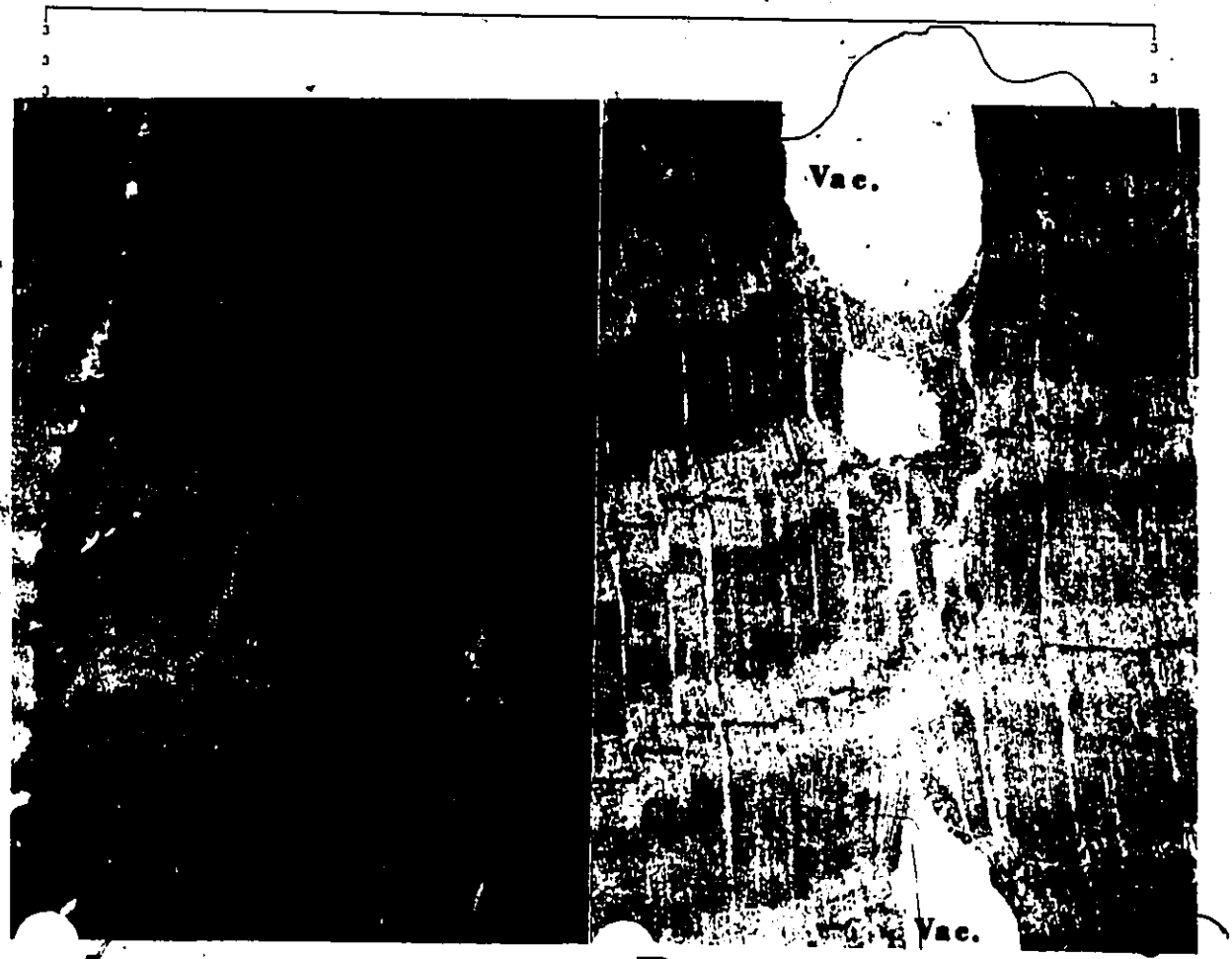

Figure 13: Longitudinal sections of muscle fibres exposed to glycerol Ringer then returned to normal Ringer for 1 hour (A) and returned to normal Ringer for 4 hours (B). In the last 30 minutes of each recovery 0.05% HRP was added. Notice that the HRP reaction product is confined to the extracelluar space. Vacuoles of varying size and distribution were seen after both 1 (A) and 4 hours (B) of recovery. The vacuoles do not contain HRP reaction product. Magnification of A 11,400x; magnification of B 13,965x.

the extracellular space even after a 4 hour recovery period in normal Ringer. It was estimated from examination of all electron micrographs of glycerol shocked muscles that over 90% of the transverse tubules were devoid of HRP in agreement with Eisenberg and Eisenberg (1968) and Franzini-Armstrong et al. (1973).

The efficacy of excitation-contraction coupling of glycerol shocked muscles was examined by electrical stimulation as described above. Paired muscles were used. One muscle of each pair was subjected to the glycerol treatment and allowed to recover for 4 hours while its companion muscle was left in normal Ringer. At the end of the glycerol shock, the untreated muscle was mounted in the recording chamber (Figure 6, ChapterVI) with a continuous flow of normal Ringer and stimulated as described above. The glycerol shocked muscle was then transferred to the recording chamber and stimulated according to the excitability curve of the untreated muscle. Invariably, the detubulated muscles showed only very small contractions and then only when stimulating voltage and duration of the stimulating impulse were greatly increased.

The electron micrographs and the electrical stimulation demonstrate that the communication between the tts and extracellular space were morphologically and electrically disconnected by the glycerol shock even after 4 hours of recovery.

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The electron micrographs and the electrical stimulation demonstrate that the communication between the tts and extracellular space were morphologically and electrically disconnected by the glycerol shock even after 4 hours of recovery.

11.3.2 ^{14}C-INULIN SPACE IN DETUBULATED MUSCLES

This experiment was performed to yield the following information about the detubulated fibres of a given muscle. First, it is imperative that the size of the extracellular space (i.e. ^{14}C-inulin space) of the 4 hour recovered muscle be determined partly to assess the muscle fibre for damage but primarily because a realistic extracellular space was required to determine the total intrafibre electrolyte content from total analysis, since an *a priori* assumption of a constant extracellular space following the glycerol treatment cannot be valid. Second, it is interesting to know if the ^{14}C-inulin that penetrates the tts could become permanently trapped in the tts when they are disconnected from the extracellular space during re-exposure of the muscle to normal Ringer.

A total of 45 muscles were used and divided into three groups of 15 muscles. In one group, the muscles were stored in normal Ringer for a total of 6 hours. The normal Ringer was changed twice at two hour intervals. During the first and the third 2 hour periods ^{14}C-inulin was included in the Ringer. This group served as controls. The second group of muscles were exposed to normal Ringer containing ^{14}C-inulin for 1 hour then transferred to glycerol Ringer with ^{14}C-inulin.These muscles were then returned to normal Ringer without ^{14}C-inulin for 4 hours to recover and to eliminate all extracellular inulin. The third group of muscles were first transferred to glycerol Ringer without ^{14}C-inulin then

returned to normal Ringer also without ^{14}C-inulin for two hours. Finally, these muscles were transferred to normal Ringer containing ^{14}C-inulin for an additional 2 hours. This group was used to assess the damage sustained during the glycerol shock and to measure the extracellular space volume of 4 hour postglycerol shocked muscles. All muscles were analyzed for water and ^{14}C-inulin content as descibed. The results are summarized in Table 13.

The control muscles (row I) gave an extracellular space of about 20% similar to other estimates by inulin (Neville, 1979; and Steinbach, 1961) and also similar to estimates by ^{3}H-sucrose (Sperelakis et al., 1978). The extracellular space of 4 hour postglycerol treated muscles (row III) also gave an extracellular space of about 20%. The close agreement between the two values indicates that glycerol treated muscles did not sustain significant damage.

More interestingly, the muscles which were exposed to ^{14}C-inulin prior to the occlusion of the tts (row II) showed that ^{14}C-inulin was retained in the tts even after 4 hours of recovery in non-radioactive Ringer. Clearly, the tts was disconnected from the extracellular space and remained so even after 4 hours. The entrapment of an extracellular space marker in the transverse tubules following glycerol shock has already been observed by Krolenko (1969) using ferritin. The calculated volume of the tts is almost 2% of the muscle water or about 10% of the extracellular space.

er estimates based on morphology could be in error if shrinkage had occurred during tissue fixation. This possibility is supported by the the dependence of transverse tubular morphology on the method of fixation (Page, 1964). The estimate by electrophysiolgy is very indirect and dependent on a number of assumptions linking the delay of membrane potential changes when the external K concentration is changed to a temporary accumulation of K in an extracellular compartment. The larger estimates of Table 13 and of Endo (1966) could be in error if the extracellular markers (^{14}C-inulin and Rhodamine Lissamine B-200) were non-specifically adsorbed on the connective tissue of the sarcolemma and on the ground substance on the the walls of the tts. The important point of the Table 13 (row II) data, however, is not the calculated size of the tts but, rather, the demonstration that ^{14}C-inulin can be trapped in the tts and remain so for 4 hours. This is very good demonstration that the tts remains occluded from the extracellular space during the recovery period. The high $\%H_2O$ of the glycerol treated muscles (rows II and III) indicates that the recovery was incomplete even after a 4 hour recovery period. This high $\%H_2O$ is consistent with the vacuolation seen in the recovered fibres (Figure 13).

Because of the increased water content , it is probable that the extracellular space volume has changed even though the inulin space appears to remain constant. If one assumes

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Table 14: CHANGES IN K-ACTIVITY AND TOTAL MUSCLE K AND Na DURING GLYCEROL SHOCK AND RECOVERY.

Solution	Em(mV)	$(aK)^1 x10^{-3}$	TOTAL MUSCLE CONCENTRATION* (K)t	(Na)t	$\%H_2O$
			(m.moles/kg muscle water)		
I Normal Ringer	-85±0.5 (85)	114±2.5 (45)	105±1.8 (37)	48±1.0 (37)	78.7±0.2 (37)
	p<0.001	p<0.001	p<0.025	p<0.001	
II Glycerol Ringer (1 hour)	-88±0.6 (80)	144±8.0 (34)	117±5.0 (8)	52±2.8 (8)	74.0±0.5 (8)
	p<0.001	p<0.001	p<0.001		p<0.001
III Normal Ringer (1 hour Recovery)	-68±1.2 (86)	100±5.0 (48)	63±1.9 (19)	53±2.8 (20)	84.4±0.2 (20)
	p<0.001	p>0.05	p<0.001	p<0.001	p<0.001
IV Normal Ringer (4 hours Recovery)	-83±1.0 (58)	112±5.0 (14)	78±1.5 (45)	63±0.9 (41)	80.7±0.2 (45)

*Values are expressed as total muscle water not corrected for extracellar space because extracellular space may vary with muscle water content.

p values are the probability that the differences between the mean above and below are zero.

However, this view is an over-simplification because gly-

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	p<0.001	p<0.001	p<0.001		p<0.001
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	p<0.001	p>0.05	p<0.001	p<0.001	p<0.001
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cerol is known to to permeate the sarcolemma (Bozler, 1961; Krolenko and Adamjan, 1967; Dulhunty and Gage, 1973). Dulhunty and Gage (1973) demonstrated that muscles exposed hypertonic glycerol Ringer shrank, indicating water loss. However, this shrinkage was only temporary and the muscle fibres recover their volume within 20-25 minutes. This suggests that glycerol had permeated the membrane and had replaced the water. To complicate matters even further, the glycerol that enters the muscle fibre will also contribute to the dry weight (the boiling point of glycerol is 290 °C while the drying temperature used in this thesis was 95 °C) thereby lowering the $\%H_2O$. Moreover, Henderson (1970) has reported that muscles lose intrafibre K during exposure to hypertonic glycerol Ringer. Thus it may be a serious oversimplification to evoke a simple water loss as the sole explanation for the increase in electrolyte content.

If there was indeed a loss of fibre K during the 1 hour glycerol (Henderson, 1970), then the increased $(aK)^i$ (row II) could result from either a release of bound K into the myoplasm or from a change in the activity co-efficient of the myoplasm as glycerol enters or even from a disproportionately large loss of myoplasmic water. Recall that about 15% of the intrafibre water of a frog sartorius muscle is osmotically inactive and that this water resides in the myofibrils. The second hypothesis was examined by measuring the K-activity (with the K-selective microelectrode) in a

150 mM KCl solution with and without 400 mM glycerol. The activity co-efficient increased from 0.737 to 0.767 by the glycerol. This increase is only 4% and, therefore, cannot account for the increase in $(aK)^1$ of more than 20%. Thus it is plausible that an internal release of of myofibrillar K might have occured or alternatively there was a disproportionately large loss of myoplasmic water.

The decreases in $(aK)^1$ and (K)t and the increase in $\%H_2O$ in the 1 hour recovered muscles (row III, Table 14) suggests that there was a swelling of the muscle fibres when the muscles were returned to normal Ringer from hypertonic glycerol Ringer. The elevated water content is consistent with Henderson (1970) who reported that glycerol treated muscles remain swollen for up to 4 hours when returned to normal Ringer but inconsistent with Dulhunty and Gage (1973) who reported that glycerol treated muscle remain swollen for only 20 minutes when returned to normal Ringer. The (K)t and (Na)t data are consistent with the findings of Bianchi and Bolton (1974) who concluded that a loss of fibre K and a gain of fibre Na had occurred. However, the fall of $(aK)^1$ during the first hour of recovery was not as low as would be predicted from the fall in (K)t. This is further evidence that there was indeed a release of bound K into the myoplasm during the glycerol treatment.

In the 4 hour recovery data (row IV), $(aK)^1$ and Em have returned to normal. The $\%H_2O$ has recovered close to its

preglycerol level, however, (K)t and (Na)t have not. These changes in $\%H_2O$, (K)t and (Na)t are in agreement with those of Henderson (1970, Table 3). These results show that even after 4 hours of recovery the muscle fibres have not recovered entirely. It is interesting that $(aK)^1$ and Em have recovered but (K)t and (Na)t have not. There are two explanations for this phenomenon. First, the postulated release of bound K into the myoplasm during the glycerol treatment may not be reversed or alternatively the changes in total muscle electrolyte content may be related to the development of vacuoles during the recovery. The latter possibility will be pursued in the Discussion of this chapter.

11.3.4 RESPONSE TO K-FREE RINGER BY DETUBULATED MUSCLES VS RESPONSE TO K-FREE RINGER BY NORMAL MUSCLES.

As discussed above, the tts may be the operative pathway by which changes in the external milieu are communicated to the fibre interior. If this reasoning is correct, then detubulated muscles should have an attenuated response to to K-free Ringer. Paired muscles were dissected from two frogs of the same batch in each experiment. One pair was identified as the control. Its response to K-free Ringer was measured as before (see Chapter VIII) to obtain the changes in free and total intrafibre K and Na. The second pair was first exposed to hypertonic glycerol Ringer for 1 hour and allowed to recover for 4 hours in normal Ringer. This pair was then handled in an identical manner as the controls.

The results are summarized in Table 15. The upper three rows are data from the control muscles and the lower three rows are from the glycerol detubulated muscles.

It must be mentioned that these experiments contained the basic flaw of being performed on frogs from a variety of sources (i.e. from various suppliers and from local ponds) from November, 1980 to December, 1981. Furthermore, most of the experiments in which the K-selective microelectrode was used were done in the summer months on frogs from local ponds whereas most of the experiments in which the Na-selective microelectrode was used were done in the fall and winter months. This wide sampling of frogs from different seasons and from different sources leads to relatively large scatter in the results. All the collected data have been pooled into Table 15 hence the reader should not attempt a critical correlation between $(aK)^1$ and Em and between $(aK)^1$ and (K)i as had been done in Chapter VII.

Despite these problems the control results (upper three rows) still reveal the typical response to K-free Ringer: hyperpolarization of the membrane potential, a fall in $(K)^1$, but a constant (K)i, and increases in both $(Na)^1$ and (Na)i. Except for the rather large fall in $(K)^1$, all the mean changes are comparable to those in Table 3 of Chapter VIII, a much more carefully controlled experiment. In contrast, the detubulated muscles do not show the typical response to the K-free Ringer treatment (compare rows VI and III). Com-

Table 15: CHANGES IN FREE AND TOTAL K AND Na DURING A 40 MINUTE EXPOSURE TO K-FREE RINGER BEFORE AND AFTER DETUBULATION

Superfusate	Em (mV)	$(K)^1$ (mM)	$(Na)^1$ (mM)	$(K)i$ (m.moles/kg f.w.)	$(Na)i$ (m.moles/kg f.w.)	$\%H_2O$
Normal Ringer (untreated muscle)	-86± 0.6 (96)	177± 6.7 (31)	22± 1.3 (49)	137± 2.5 (18)	26± 1.2 (20)	78.4± 0.2 (20)
K-free Ringer (untreated muscles)	-102± 1.0 (90)	123± 8.0 (25)	37± 2.1 (49)	136± 2.5 (19)	32± 1.3 (20)	78.0± 0.2 (18)
Difference (untreated muscles)	-16± 1.2 (184) p<0.001	-54± 1.0 (54) p<0.001	+15± 2.5 (96)	-1± 3.5 (35)	+6± 1.8 (38) p<0.001	-0.4± 0.3 (36) p<0.001
Normal Ringer (post-glycerol)	-70± 1.6 (107)	144± 6.7 (51)	32± 2.4 (36)	99± 2.5 (49)	51± 1.6 (37)	80.7± 0.1 (70)
K-free Ringer (post-glycerol)	-67± 1.7 (129)	140± 6.7 (65)	28± 2.0 (44)	95± 2.5 (37)	52± 1.6 (37)	80.4± 0.1 (68)
Difference (post-glycerol)	+4 ± 2.3 (234) p>0.2	-4 ± 9.5 (114) p>0.5	-4 ± 3.5 (91) p>0.5	-4± 3.1 (71) p>0.2	+1± 2.3 (72) p>0.5	-0.3± 0.1 (136)

parison of the row IV Table 14 data with the data of Table

15 shows that the Em of detubulated muscle fibres do not always recover, however, the abolition of the response to K-free was never dependent on the recovery of the Em. Clearly, the response to K-free Ringer treatment has been abolished by some change or changes produced by the detubulation procedure.

11.4 DISCUSSION

Krolenko (1969) observed that vacuoles appeared in glycerol treated muscles when they were returned to normal Ringer. These vacuoles were often observed at the level of the Z-line (see also Figure 13) and to take up ferritin when ferritin was included in the recovery solution. The data of Table 13 shows that the tts can entrap previously deposited ^{14}C-inulin when glycerol treated muscles are returned to normal Ringer. Presumably, most of the transverse tubules enlarge into vacuoles after being occluded from the extracellular space. Bianchi and Bolton (1974) found that the ^{3}H-inulin space of glycerol treated muscles doubled during the first hour of recovery when ^{3}H-inulin was included in the recovery solution of normal Ringer. They attributed this increase in the ^{3}H-inulin space to damaged fibres. However, the ^{14}C-inulin space of 4 hour postglycerol muscles was only about 20% (Table 13) indicating that glycerol shocked muscles do not sustain significant damage. The valuof Table 13 (row III) was acquired after to the occlu-

sion of the transverse tublues and, therefore, does not include the vacuolated tubules. The ^{3}H-inulin space measured by Bianchi and Bolton (1974) was acquired during the occlusion of the transverse tubules and would presumably included at least part of the volume of the vacuolated tubules. If the increase in the ^{3}H-inulin space observed by Bianchi and Bolton (1974) were entirely due to vacuolation, which if assumed to contain extracellular fluid lead to the conclusion that the intrafibre water of glycerol treated muscles must be over-estimated by the method of its calculation in this thesis. It was assumed that the total intrafibre water was the total analyzed water minus about 20% due to the extracellular space. It follows that caculations of the total intrafibre concentrations of K and Na in glycerol shocked muscles may also be in error since these concentrations were calculated with the same assumption. If the vacuoles did indeed double the extracellular space from 20% to 40% of the analyzed water then the actual intrafibre water content would only be 60% of the total analyzed water. The "corrected" total intrafibre concentration of K and Na (row III, Table 14) are 128 and 28 m.moles/kg fibre water respectively. These valuesare very similar to those of normal muscles (row 1, Table 14). Perhaps, the changes in the total muscle K and Na during the glycerol shock may reflect changes in the volume of the transverse tubular system. This, however, cannot explain the changes to $(aK)^1$ during

the glycerol shock. Dulhunty and Gage (1973) have postulated that vacuolation may be caused by a shift of intrafibre water into the transverse tubules. This can explain the recovery of $(aK)^1$ from 1 hour to 4 hours postglycerol treatment (compare rows III and IV, Table 14) but not the changes in total intrafibre K and Na concentration if one assumes that water is but that K is not allowed to permeate the tubular membrane. However, this is unlikely since the tubular membrane is highly permeable to K (Eisenberg and Gage, 1969). Clearly, the glycerol treatment has produced some interesting but inexplicable changes in the distribution of monovalent cation in the frog sartorius muscle.

Since, the transverse tubules have been disconnected from the extracellular space both morphologically and electrically, it is tempting to conclude that the abolition of the response to K-free Ringer was due specifically to the elimination of the transverse tubular system as the pathway by which changes in the external electrolyte concentration are detected by the myofibrils. Unfortunately, this conclusion must remain tentative because other pertinent alterations might have occurred which may have also abolished the response to K-free Ringer. For example, Henderson (1970) and Venosa and Horowicz (1973) observed subtle alterations to the sarcolemma following a long recovery period from glycerol treatment. More important, perhaps, was the observation of Bianchi and Bolton (1974) who found that the glycerol treatment produced a drop in muscle Mg concentration

which cannot be accounted for by assuming that the fall in Mg concentration was due to the appearance of vacuoles filled with extracellular fluid. In Chapter (IX) it was suggested that Mg may play a key role in the K-free Ringer induced internal K-shift. It is possible that any loss of intrafibre Mg could have altered the internal redistribution of K. The one safe conclusion that can be drawn from the data of this chapter is that detubulation produced alterationsthat have abolished the ability of the muscle fibre to internally sequester free intrafibre K when exposed to K-free Ringer.

Chapter XII

INTRAFIBRE CONSERVATION OF K BUT NOT NA

12.1 INTRODUCTION

During acute exposures to K-free Ringer, there was an internal sequestration of free K rather than a transmembrane efflux so that total intrafibre K was conserved even at the expense of free K (at least for 1 hour). This conservation of intrafibre K is retained even when the K-free Ringer induced internal K sequestration is abolished by removal of external Na or by detubulation. In contrast, intrafibre Na (both free and total) is rapidly lost during Na-free Ringer treatment (White and Hinke, 1976). Vaughan-Jones (1977) also observed a rapid fall of $(aNa)^1$ in crab muscle fibres exposed to low Na Ringer (1/10 normal Na). This fall in $(aNa)^1$ was insensitive to ouabain ($2x10^{-4}$ M) and to Na/Ca exchange blockers (D-600 and Verapamil). This led Vaughan-Jones to postulate that the rapid fall in $(aNa)^1$ was caused either by a membrane ion-exchange mechanism or by an internal redistribution of myoplasmic Na to a location not detected by the Na-selective microelectrode. Unfortunately, he did not carry out chemical analysis to test his hypotheses. However, as described by White and Hinke (1976) who combined the Na-selective microelectrode with ^{22}Na efflux, intrafibre Na is indeed lost.

The purpose for carrying out these Na-free Ringer experiments and to include them in this thesis was to contrast the response of intrafibre K and Na when each is eliminated from the surrounding medium.

The experiments in this chapter are an extension of experiments reported by White and Hinke (1976).

12.2 RESULTS

12.2.1 CONTINUOUS RECORDINGS OF $(aNa)^1$ IN Na-FREE RINGER

Continuous recordings were carried out on single surface fibres while the superfusate was changed from normal Ringer to Na-free Ringer. This experiment is identical to one described by White and Hinke (1976). The results of six successful experiments are summarized in Figure 14.

The decline in $(aNa)^1$ was rapid and comparable to that of White and Hinke (1976) and of Vaughan-Jones (1977) (i.e. stabilizing at about 3×10^{-3} after about 40 minutes). This fall in $(aNa)^1$ can be fitted by $(aNa)^1 = 7.2 \exp -0.127(t-2) + 3.3$ where t is in minutes. This experiment shows that free intrafibre Na disappears rapidly when the muscle is exposed to Na-free Ringer.

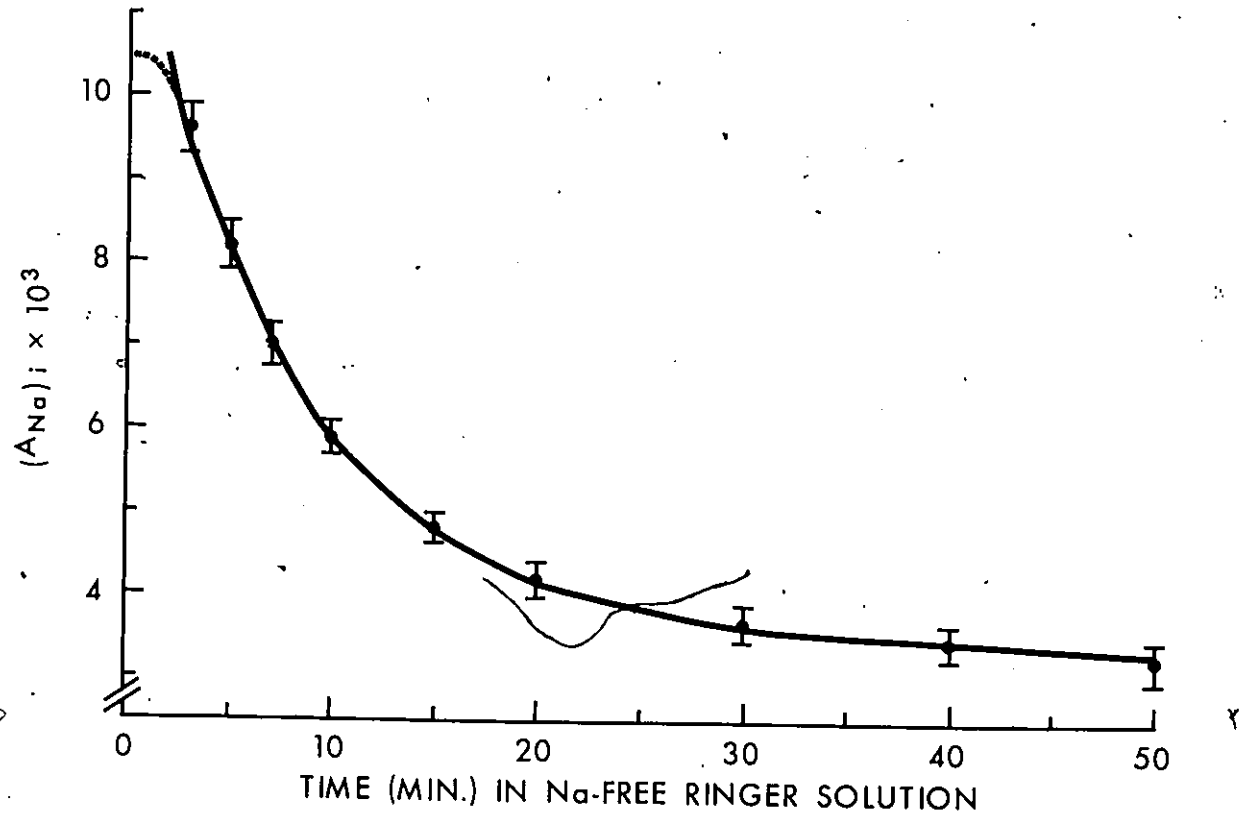

Figure 14: Averaged continuous recordings of $(aNa)^1$ from 6 experiments when the superfusate is changed from normal Ringer to Na-free Ringer. The curve is described by the equation $(aNa)^1 = 7.2\exp(-0.1279t - 2) + 3.3$. The bar denotes ± 1 standard error of the mean.

12.2.2 CHANGES IN FREE AND TOTAL Na DURING Na-FREE RINGER TREATMENT.

This experiment was performed to determine the concomitant fall in total intrafibre Na by chemical analysis rather than by isotopes (White and Hinke, 1976) when free Na exits the muscle fibre into the extracellular space during Na-free Ringer treatment. Paired muscles were used. The protocol was identical to the K-free Ringer treatment protocol of Chapter VIII except Na-free Ringer replaced normal Ringer instead of K-free Ringer. The results are summarized in the upper three rows of Table 16. The results of a larger group of muscle in which only chemical analysis was done are included in the bottom three rows. Both groups of muscles show an identical loss of total intrafibre Na even though one set of muscle was exposed to Na-free Ringer for 30 minutes and the other set for 40 minutes.

These Na losses confirm the conclusion of White and Hinke (1976) that the rapidly disappearing free Na traverses the plasma membrane into the extracellular space. The total intrafibre K data (bottom three rows, Table 16) suggests that extracellular K may be exchanging with intrafibre Na in a 2:1 coupling ratio as Na leaves the muscle fibre. However, this change is not statistically significant.

Table 16: CHANGES IN FREE AND TOTAL INTRAFIBRE K AND Na AFTER A 30 MINUTE AND AFTER A 40 MINUTE EXPOSURE TO Na-FREE RINGER

Superfusate	$(Na)^1$ (mM)	(Na)i (m.moles/kg f.w.)	(K)i (m.moles/kg f.w.)
Normal Ringer	15.3±0.5 (10)	24.1±2.0 (12)	
Na-free Ringer (30 min.)	4.1±0.2 (10)	10.6±1.0 (10)	
Difference	-11.2±0.5 (18) $p<0.001$	-13.5±2.2 (10) $p<0.001$	
Normal Ringer		23.0±2.0 (36)	147±2.0 (34)
Na-free Ringer (40 min.)		9.4±0.4 (34)	153±1.6 (37)
Difference		-13.6±2.0 (68) $p<0.001$	+6±2.6 (37) $0.1<p<0.2$

12.2.3 CONTINUOUS RECORDINGS OF $(aNa)^1$ AND $(aK)^1$ IN Na-FREE RINGER VS Na-RINGER + 10^{-4} M OUABAIN.

This experiment was carried out to determine whether the efflux of Na during Na-free Ringer treatment was via the Na/K pump as suggested by White and Hinke (1976). Either $(aNa)^1$ or $(aK)^1$ were recorded continuously in surface fibres when the superfusate was changed from normal Ringer to

either Na-free Ringer or to Na-free Ringer with 10^{-4} M ouabain. The fall of $(aNa)^1$ in Na-free Ringer with ouabain is shown in Figure 15 curve A (curve B is replotted from Figure 14 for comparison). The increase in $(aK)^1$ during Na-free Ringer superfusion is shown in curve A of Figure 16 and the fall in $(aK)^1$ during superfusion with Na-free Ringer containing 10^{-4} M ouabain is shown in curve B of Figure 16.

The data of Figure 15 is similar to that of White and Hinke (1976), that is, ouabain inhibited but did not abolish the efflux of $(aNa)^1$. After 50 minutes of superfusion, $(aNa)^1$ in the Na-free Ringer with ouabain was about twice that in Na-free Ringer. Thus one may conclude that part of the Na loss is through the Na/K pump.

Curve A of Figure 16 illustrates that the increase of $(aK)^1$ in Na-free Ringer reached a maximum after about 10 minutes and began to decline after about 20 minutes. The maximum increase in $(aK)^1$ (about 7×10^{-3}) is equivalent to the fall of $(aNa)^1$ after 40 minutes. The magnitude of these changes suggests that Na left the muscle fibre in a 1:1 exchange with external K. However, caution must be exercised when one is making use of activity measurements in this manner because an internal exchange myofibrillar Na for myoplasmic K may also be occurring simultaneously. Certainly, the transient increase in $(aK)^1$ can be used as supporting evidence for such an internal exchange. That is, the transient increase in $(aK)^1$ may have been caused first by a

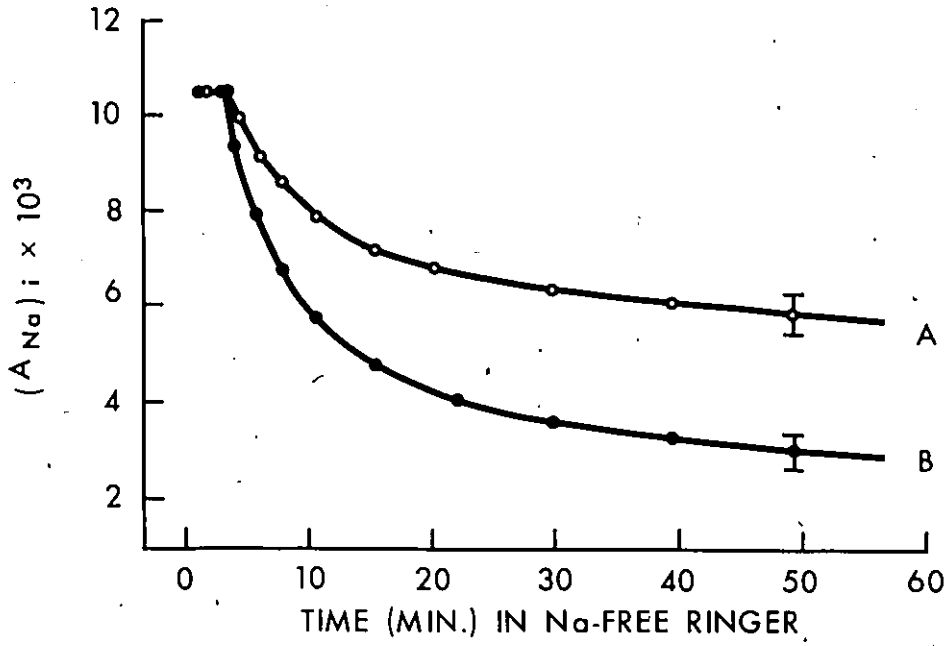

Figure 15: Average continuous recordings of $(aNa)^1$ in Na-free Ringer containing 10^{-4} M ouabain from 6 experiments (curve A). Curve B is replotted from Figure 1 for comparison.

transmembrane exchange of K and Na during the first 20 minutes which was then followed by an internal exchange during the next 20 minutes.

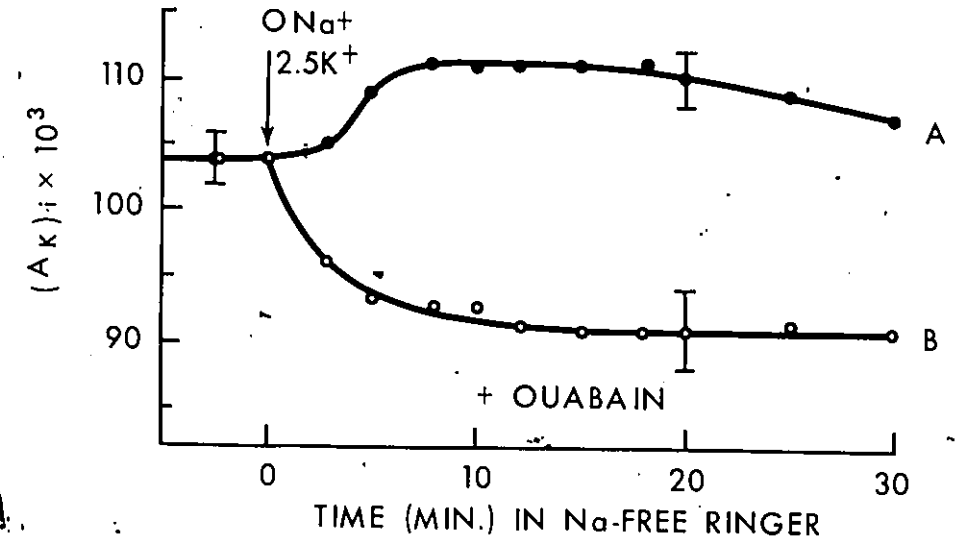

Figure 16: Averaged continuos recordings of $(aK)^1$ in Na-free Ringer (curve A) from 15 experiments and in Na-free Ringer containing 10^{-4} M ouabain (curve B) from 12 experiments.

Curve B of Figure 16 illustrates that ouabain blocked the increase of $(aK)^1$ during the Na-free Ringer treatment indicating that the K:Na exchange was indeed linked by the Na/K pump. In fact, ouabain did more than just to block the

pathway by which K exits the muscle fibre remains to be shown.

12.3 DISCUSSION

This chapter clearly shows that Na rapidly leaves the muscle fibre when the muscle is exposed to Na-free Ringer in contrast to the conservation of total intrafibre K when the muscle is treated with K-free Ringer. Part of this Na-efflux is ouabain sensitive and linked to an influx of K through the Na/K pump. The fact that both $(aNa)^1$ and $(Na)i$ fell when the Na/K pump was inhibited by ouabain shows that Na can exit the muscle fibre by a pathway other than the Na/K pump. Likewise, the loss of K when Na is also being lost (Na-free Ringer+ouabain) shows that K can leave the muscle fibre independent of Na movements through the Na/K pump. These observations are not new. They do, however, illustrate the complex ionic movements that can occur when the normal ionic distribution is perturbed.

The observation that the $(aK)^1$ increase in Figure 16 was only temporary and the observation that $(aK)^1$ and $(K)i$ were not equal after 40 minutes (compare curve A, Figure 16 with Table 16) is further evidence that intrafibre K is not uniformly distributed. It was suggested that the temporary increase in $(aK)^1$ was caused by an internal exchange of K for Na. Likewise, the non-uniform distribution of Na is corroborated by the two phase decline of $(aNa)^1$ in Na-free

Na/K pump, it initiated a loss of free intrafibre K. The fact that K and Na can both leave the muscle fibre when the Na/K pump is blocked by ouabain shows that K and Na must be able to cross the membrane by pathways other than the ouabain sensitive Na/K pump.

To confirm that ouabain did initiate a loss of K to the extracellular space, chemical analysis was carried out on a group of muscles exposed to Na-free Ringer containing 10^{-4} M ouabain. The data is summarized in Table 17. The data in Table 17 clearly show that the free K does leave the muscle fibre into the extracellular space. However, the

Table 17: CHANGES IN TOTAL K AND Na AFTER A 40 MINUTE EXPOSURE TO Na-FREE RINGER CONTAINING 10^{-4} M OUABAIN.

Superfusate	(Na)i (m.moles/kg f.w.)	(K)i (m.moles/kg f.w.)
Normal Ringer	20.5±2.0 (24)	156±1.5 (22)
Na-free Ringer + 10^{-4} M Ouabain	12.7±0.4 (37)	144±1.9 (43)
Difference	-7.8±2.0 (59) $p<0.001$	-12±2.4 (63) $p<0.001$

Ringer. A two compartment analysis similar to the one carried out in Chapter VIII was not attempted for the the data of this chapter because the parameters required were not acquired in properly controlled experiments and because the change in (K)i (Table 16) was not statistically significant.

Another interesting revelation from the data of this chapter and the data of Chapter X is that the muscle fibre can lose large amounts of intrafibre Na rapidly when exposed to Na-free Ringer or to K-and Na-free Ringer. This observation is of importance to studies of monovalent cation transport where a 10 to 30 minute wash in Na-free or K-and Na-free Ringer have been used to remove Na and K from the extracellular space. For example, Sjodin and Beauge (1973) used a Na-free Ringer wash to determine the intrafibre Na concentration and found that a 30 minute wash gave a lower concentration than a 10 minute wash (16.9 m.moles/kg fibre water vs 10.6 m.moles/kg fibre water). It is worth mentioning that their values are lower than those obtained by investigators who have not used this technique (see Lev, 1964; Lee and Armstrong, 1974 and this thesis). The underlying assumption is that the intrafibre ionic content is not altered by such a brief wash when in actual fact free intrafibre Na leaves the muscle fibre with a rate constant comparable to the extracellular clearance of ^{22}Na (White and Hinke, 1976; and Neville, 1979). This chapter provides unequivocal evidence that this practice can no longer be acceptable.

Chapter XIII

SIGNIFICANCE OF THE RESULTS

There are several new and important observations in this thesis. Perhaps, the most important and certainly the most interesting is the observation that free intrafibre K fell when the muscle was exposed to K-free Ringer yet total intrafibre K remained constant. Application of the two compartment model revealed that this disappearance of free intrafibre K was mediated by an internal sequestration of free K by the myofibrils. In addition, the two compartment model reveals that free Na was also sequestered during the exposure to K-free Ringer and that the sequestration of the monovalent cations was an exchange process for some as yet undetermined myofibrillar cation probably Mg. It is also interesting that the internal sequestration of free K can be abolished by removing the external Na or by detubulation. Together these results suggest that perhaps the entry of Na across the tubular membrane may have in some way initiated the internal sequestration of free K. These results also show that the frog skeletal muscle has in some inexplicable manner developed a means for conserving its total intrafibre K when K is acutely removed from the external milieu.

As a point of contrast to the K-free Ringer treatment, the data of Chapter XIII show that muscles exposed to Na-free Ringer lost both free and total intrafibre Na rather rapidly. Clearly, the frog skeletal muscle has developed a means of conserving its fibre K in K-free Ringer but has not developed a corresponding means of conserving its Na when Na is acutely eliminated from the external milieu. The rapid loss of fibre Na in either Na-free Ringer or in K-and Na-free Ringer also has a point of technical significance. The fact that both free and total Na are rapidly lost (free Na is in fact lost at a rate comparable to the extracellular clearance of Na) during Na-free or K-and Na-free Ringer treatment obviates the assumption that the treatment of a muscle with either of these solutions to remove extracellular Na and/or K will not significantly alter the intrafibre distribution of K and Na. Clearly, the results acquired by this type of protocol require careful re-evaluation.

Another interesting observation revealed by the two compartment analysis was the observation that ouabain treatment and K-free Ringer treatment have distinctly different effects on the intrafibre distribution of monovalent cations. There is no doubt that both treatments will blocked the normal operation of the Na pump. This point is not in contention here (recall that both treatments produced very similar increases to the total intrafibre Na concentration, see Chapter VIII). However, the data clearly show

that K-free Ringer inducedan internal sequestration of free K and to a minor extent an internal sequestration of Na probably by the myofibrils in exchange for Mg; whereas ouabain treatment induced an internal exchange of free Na for myofibrillar K. In fact it was shown in Chapter VIII that both treatments operated independently and antagonistically with repect to K. That is, during treatment with K-free Ringer containing 10^{-4} M ouabain the fall in free K was produced by a ouabain induced transmembrane efflux of K and by a K-free Ringer induced myofibrillar uptake of free K.

The analysis of the ouabain data also revealed that only 23% of the intrafibre water acted as solvent for the free cations. The remaining 77% was by necessity of size, placed in the myofibrils. This value of "bound" water is large in comparison with estimates by other techniques. This, however, should not be interpreted by the reader to mean that 77% of the intrafibre water does not act as solvent nor should it be interpreted to mean that this water is some state significantly different from that of pure water. It is impossible to confer some unique thermodynamic state to this water, in fact it has already been argued that the myofibrillar water cannot be so tightly bound to the myofilaments as to lose its Brownian motion. All that one can conclude from this analysis is that the myofibrillar water has in some way been altered so that it does not act as solvent for that fraction of ions measured by the ion-selective microelectrode.

effects of K-free Ringer are vastly different from long term effects. It is interesting that the acute effects of K-free Ringer is an internal exchange of free K for some unknown cation whereas the long term effects is an internal exchange of free Na for bound K. This observation suggests that between 1 and 24 hours of immersion in K-free Ringer some intrafibre change has taken place such that the internal ionic exchanges are now modified. Perhaps the intrafibre change is in some manner related to an alteration of contractile protein structure that may occur after dissection.

Another interesting observation of this thesis was the seasonal variation in the free and total K concentrations. Admittedly, no rigorous treatment of this data is possible since it was acquired from frogs purchased from several suppliers and from wild frogs caught locally. It does, nevertheless, show that winter frogs have the highest total intrafibre K but the lowest free K and that the converse holds true for the summer frogs. This implies that the level of K binding is also seasonally variable. The observation that the intrafibre electrolyte concentration varies with season and the observation that the electrolyte concentration also varies from frog to frog illustrates the importance of using a strict set of controls in experiments where changes to the electrolyte concentration is expected.

The experiments of this thesis began as an extension of the work of Armstrong and Lee (1971) and Lee and Armstrong

What is the significance of the data in this thesis? The data confirm the conclusion that monovalent cations are not distributed homogeneously in a muscle fibre and that the compartmentalized monovalent cations are located in the myofibrils. These results also demonstrate that the membrane alone does not determine the intrafibre distribution of monovalent cations. In fact during the 40 minute K-free Ringer treatment the fate of K was dominated by intrafibre events. Moreover, the intrafibre events during the 40 minute K-free Ringer treatment appears to have produced profound effects on the muscle fibre such that when the muscle is returned to normal Ringer there was an uptake of Na and a loss of intrafibre K rather than the expected loss of intrafibre Na and uptake of K when the Na/K pump is re-activated. This discussion is not meant as a challenge to the membrane theory, it is merely meant to illustrate that compartmentalization can and indeed does modify the response of the muscle fibre to changes in the external milieu. This conclusion clearly demonstrates that when membrane ion transport characteristics are to be studied rigorously, activity measurements must be made.

It was suggested in Chapter IV that there was doubt that the long term effects of K-free Ringer could be extrapolated to the first hour of solution change. Comparison of the data in this thesis and the data of Armstrong and Lee (1971) and Lee and Armstrong (1974) clearly shows that the acute

(1974). The results presented confirm their work that K is not homogeneously distributed in a muscle fibre. Furthermore, this thesis has shown that the myofilaments are responsible for the heterogeneous distribution. It has also been demonstrated that the long term effects of K-free Ringer cannot be extrapolated back to the first hour of K-free Ringer treatment. The most important conclusion in this thesis is that perturbations of the normal ionic distribution in a frog skeletal muscle will invoke intrafibre ionic movements in addition to the transmembrane ionic movements. Clearly, intrafibre comparmentalization of monovalent cations plays a role in determining the ultimate intrafibre distribution of monovalent cations; a role that cannot be ignored if one is to attain a complete understanding of cellular ionic regulation.

Chapter XIV

SUGGESTIONS FOR FUTURE WORK

Application of the two compartment model to the K-free Ringer results indicated that a third ion was involved in the internal exchange of free K and Na . It was suggested that Mg might be the third cation, however, this hypothesis must be substantiated.

If free K and Na did exchange for myofibrillar Mg, then intrafibre Mg activity should increase as discussed in Chapter IX. Since a suitable Mg-selective microelectrode is now available (Lopez et al., 1983) Mg activity could be measured. The increase in Mg activity may not be as large as that predicted by the two compartment model since Mg can become bound to myoplasmic constituent such as ATP and creatine phosphate. Nevertheless, if Mg is indeed involved an increase in Mg activity should be detected.

The identity of the second compartment or possible additional compartments which play a role in the intrafibre redistribution of ions can be examined with the electron microprobe. This technique allows the determination of elemental concentrations at specific areas in a muscle fibre and thus can be used to identify the compartment or compartments involved in the redistribution of ions. Paired mus-

cles should be used for these experiments. One muscle would serve as the control while its companion muscle would be exposed to a solution designed to perturb the normal ionic distribution. Cross sections rather than longitudinal sections of the muscles should be used so that the myofibril can be examined without interference from the sarcoplasmic reticulum.

The retention of total intrafibre K during K-free Ringer treatment in this thesis disagrees with the K-depletion observed during long term exposures to K-free Ringer. This difference may be partly dependent on temperature since K-free Ringer treatment in this thesis were always carried out at room temperature while long term exposures were always carried out at about 3 °C. It is of importance to examine the relation between the intrafibre sequestration of free K during short exposures to K-free Ringer with the eventual loss of total intrafibre K during extended exposures to K-free Ringer. It is obvious that, during extended exposures to K-free Ringer, the sequestered free K must be released again if total intrafibre K is to be eventually lost. The work of Simon et al. (1957) suggests that the change from the acute events to the long term events occurs at about 8 hours of K-free Ringer treatment. Perhaps by studying the time and temperature dependence of this response it may be possible to gain some insight into the mechanism of the K-free Ringer induced intrafibre sequestration of free K.

Another interesting question that should be pursued is the apparent selective retention of K. This is even observed in muscle fibres which have been rendered leaky. Preliminary experiments by Dr. J.A. Hinke using nystatin (a reversible ionophore) have shown that muscles with a porous sarcolemma selectively retains its K but not its Na when placed in solutions which should deplete both K and Na. This indicates that perhaps the muscle fibre has an internal means of selectively retaining K not previously observed. This view is supported by the selective loss of Na in Na-free (Li substituted) Ringer (Allen and Hinke, 1970).

The question of two (or more) distinct sets of intrafibre binding sites for monovalent cations (Chapter X) arises from the observation that Li selectively displaces Na. The evidence for this hypothesis are (i) the specific displacement of Na by Li (Allen and Hinke, 1970); and (ii) the variability of the fraction of bound ions with the method of experimentation. Caille and Hinke (1972) measured the fraction of bound Na by diffusion and found that 22% of the intrafibre Na was bound. In contrast Allen and Hinke (1970) found that the fraction of bound Na measured by isotope exchange was 50% of the intrafibre Na. As pointed out by Caille and Hinke (1972 and 1973) the fraction of bound ions measured by diffusion exchanges rapidly with the free fraction with a half time of microseconds whereas the fraction of bound ions measured by isotope exchange has a half-time

of about $37hr^{-1}$. Despite the fact that the isotope exchange rate may be dependent on the membrane, it is still possible that it identifies a different type of Na binding. The possibility of multiple types of ion binding sites is supported by the titration curves of Lewis and Saroff (1957) for isolated contractile proteins.

The most pressing problem of all to be solved, however, is the unraveling of the communication signal between the fibre exterior and the myofibrils which results in an internal sequestration of free K by the myofibrils when external K is absent. Thus far, I have shown that the external Na must be present and that a patent transverse tubular system may be required. It was also suggested that the internal K sequestration may in some way be dependent on the specific entry of Na into the myofibrils via the transverse tubules. This hypothesis should be examined by exposing muscles to K-free Ringer only that the Na entry should be blocked by tetrodotoxin.

APPENDIX A

Chloride-selective microelectrodes made from Corning liquid ion-exchanger 477315 respond to a variety of small anions (Walker, 1971). In fact this ion-exchanger is better suited for fabricating iodide and bromide electrodes than for Cl electrodes. However, in amphibian skeletal muscles only bicarbonate may cause errors in estimating Cl activities. The selectivity co-efficient has been measured by various authors (Walker and Brown, 1970; Walker, 1971; Saunders and Brown, 1974; Bolton and Vaughan-Jones, 1977; and Fong and Hinke, 1981). The Cl-selective microelectrodes used in this thesis were made from the same ion-exchanger as that of Fong and Hinke (1981). The effects of bicarbonate on the calibration curve of their Cl-selective microelectrode is shown in Figure 17.

In this thesis, Hepes buffer was used instead of HCO_3/CO_2. Under these conditions, if one assumes that atmospheric carbon dioxide is in equilibrium with the Ringer and the muscle fibre then the intrafibre bicarbonate concentration was about 3mM (using an intrafibre pH of 7.2). The selectivity co-efficient of Cl over HCO_3 at a Cl activity of 5×10^{-3} is 0.1 (Fong and Hinke, 1981). Using these values,

the over-estimation of free Cl concentration caused by HCO_3 would be about 0.3mM. The measured value of free Cl concentration (Chapter IX) was about 5mM. Thus the Cl-selective microelectrode should give reliable measurements of intrafibre Cl activity.

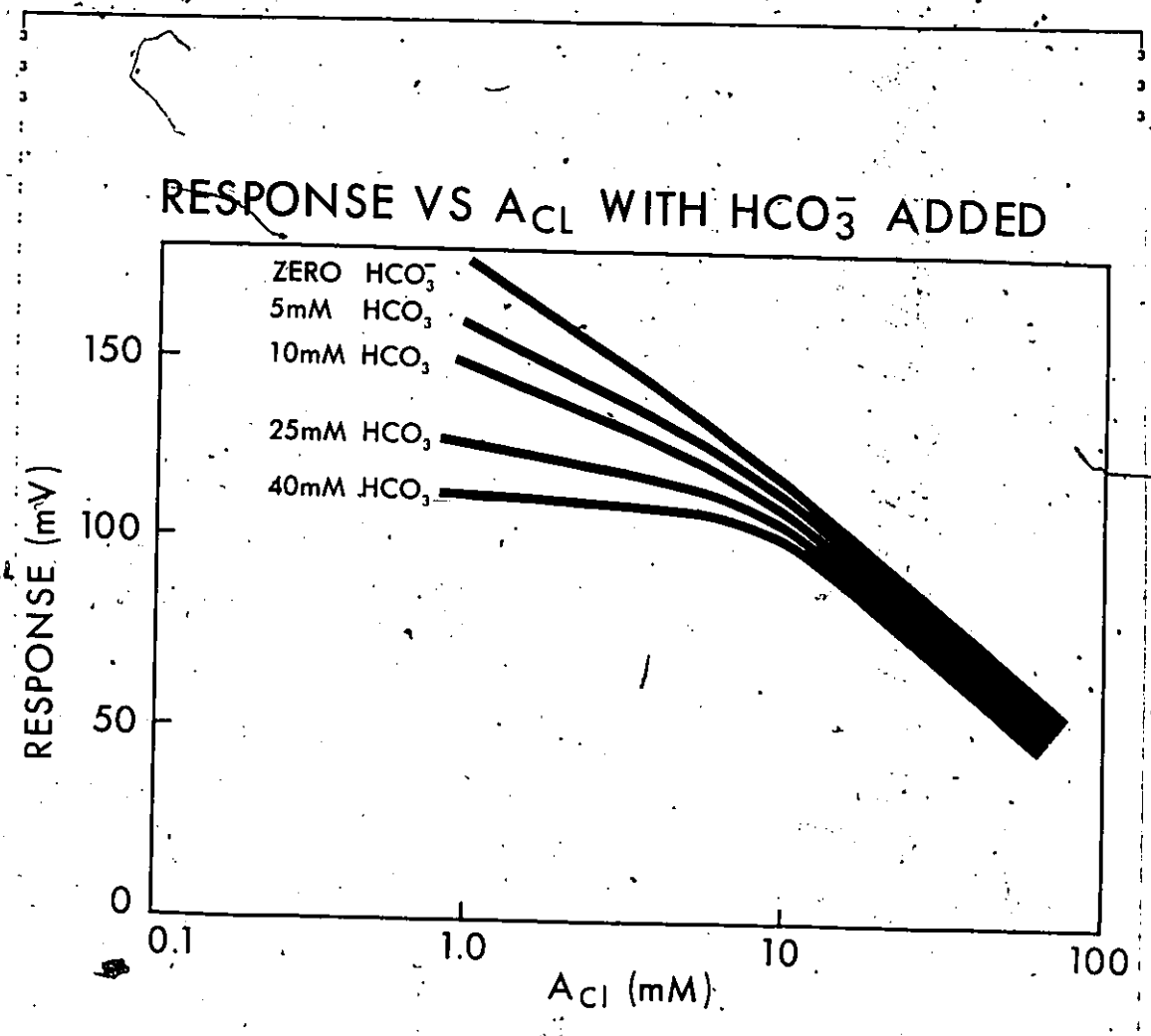

Figure 17: The effect of bicarbonate on the measured potential of the Cl-selective microelectrode (ordinate) vs. the Cl activity (abscissa). The bicarbonate is expressed as concentration. Notice that the deviation of the of the electrode response from an ideal Nernstian electrode increases as the chloride activity decreases. This deviation is exacerbated by bicarbonate.

APPENDIX B

This appendix will provide proof that the discrepancy between the loss of free K measured by the K-selective microelectrode in single surface fibres and the constant total intrafibre K measured by atomic absorption in whole muscles during the 40 minute K-free Ringer treatment was not caused by delays in the extracellular space nor by the retention of residual amount of K in the extracellular space of the deep fibres which may stimulate the Na pump of the deep fibres to re-accumulate the lost K. The efflux of a variety of solutes from the extracellular space of the frog sartorius muscle has been studied by Neville (1979). The efflux was consistent with two extracellular compartments. One compartment termed component A occupied 14% of the muscle volume. Its rate constant for ^{42}K-efflux was $1.26min^{-1}$ at $25^{o}C$ and could be predicted from the diffusion co-efficient. The second compartment termed component B occupied 9% of the muscle volume. Its rate constant for ^{42}K-efflux was $0.12min^{-1}$ at $25^{o}C$, although this rate constant was about ten times slower than that of compartment A, its rate constant was still proportional to the diffusion co-efficient.

Let us assume: (i) that all intrafibre K is free and distributed homogeneously throughout the muscle fibre: and (ii) that the two components of the extracellular space are in series such that for intrafibre K to leave the muscle proper to the bulk extracellular solution it must pass sequentially through component B then component A. These assumptions produce a model where any diffusional delays in the extracellular space will cause the greatest error between surface measurements from single fibres and whole muscle measurements.

When a muscle is exposed to K-free Ringer, K must first leave the extracellular space. As the extracellular space becomes depleted of K, intrafibre K will then leave the muscle fibre. Since this is a series model, as K leaves the extracellular space it can be replenished by intrafibre K, therefore, the total muscle K, (K)(t), at time t can be written as

$$(K)(t)=\alpha(t)\exp(-k_{\alpha}t)+\beta(t)\exp(-k_{\beta}t)+\gamma^{0}\exp(-k_{\gamma}t) \qquad (1)$$

where $\alpha(t)$ and $\beta(t)$ are the total K contents of component A and component B of the extracellular space respectively, γ^{0} is the initial total intrafibre K content and K_{α}, K_{β} and K_{γ} are the rate constants for the loss of K from component A, from component B and from the muscle fibre respectively. According to our model intrafibre K can replenish the K of

component B which in turn can replenish the K of component A, therefore, equation (1) may be re-written as

$$(K)(t)=(\alpha^0+Y)\exp(-K_\alpha t)+(\beta^0+Z)\exp(-K_\beta t)+\gamma^0\exp(-K_\gamma t) \quad (2)$$

where α^0 and β^0 are the initial total K contents of components A and B respectively and Y and Z are defined as the loss of K from component B into component A and Z is defined as the loss of intrafibre K into component B. From these definitions then

$$\gamma=(\beta^0+Z)-(\beta^0+Z)\exp(-K_\beta t) \quad (3)$$

$$Z=\gamma^0-\gamma^0\exp(-K_\gamma t) \quad (4)$$

substituting equations (3) and (4) into equation (2) gives

$$K(t)=\left\{\alpha^0+(\beta^0+\gamma^0-\gamma^0\exp(-K_\gamma t)-(\beta^0\ \gamma^0-\gamma^0\exp(-K_\gamma t))\exp(-K_\beta t)\right\}\exp(-K_\gamma t)$$
$$+\left\{\beta^0+\gamma^0-\gamma^0\exp(-K_\gamma t)\right\}\exp(-K_\beta t)$$
$$+\ \gamma^0\exp(-K_\gamma t) \quad (5)$$

where all terms have been defined. The values of K_α and K_β from Neville (1979) are 1.26min^{-1} and 0.12min^{-1} respectively and the value of k_γ from Chapter VIII is 0.008min^{-1} (0.48hr^{-1}). The

values of α^0 and β^0 calculated from Neville (1979) are 0.35 and 0.23m.moles/Kg muscle water respectively and the value of γ^0 calculated from winter frogs is 116m.moles/Kg muscle water. If we now apply these values to our model, the total muscle K after 40 minutes in K-free Ringer would be 84.5 m.moles/Kg muscle water if K did indeed leave the muscle fibre. In comparison if we now assume that no diffusional delays occur whatsoever in the extracellular space then the total muscle K after 40 minutes in K-free Ringer would be 84.2m.moles/Kg muscle water. This calculation shows that diffusional delays in the extracellular space do not lead to a retention of extracellular K at least after 40 minutes, therefore, surface measurements of single fibres do reflect changes in the whole muscle after 40 minutes. This calculation is in agreement with the observation of Henderson (1970) that the ^{42}K-efflux from whole muscles are not very different from single muscle fibres. Since K has been shown to be cleared adequately from the extracellular space after 40 minutes we can conclude that the discrepancy between the surface measurements by the K-selective microelectrode and the whole muscle measuremtns by atomic absorption spectrophometry was not caused by diffusional delays nor by the possibility that the retention of total intrafibre K may be caused by a residual low level of K around the deep fibres which stimulates the Na pump of the deep fibres to re-accumulate

the lost K. Enough supporting evidence has been given to conclude that the discrepancy between the changes in free K concentration and the total intrafibre K concentration during the 40 minute K-free Ringer treatment are real and not caused by geometric differences between the K-selective microelectrode measurements and the atomic absorption spectrophotometry measurements.

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