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ERRATUM: For sartorious read sartorius

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ABSTRACT

A new technique for direct measurement of net K^+ flux between frog sartorious muscle fibers and the external solution has been developed. This method was found to yield efflux values for the resting and active state comparable with those determined by other investigators employing radioactive tracer techniques.

Upon stimulation to fatigue the rate of net K^+ efflux was found to be double the resting efflux. When the buffering capacity of the external solution was increased by raising its bicarbonate concentration the net K^+ efflux following fatigue was observed to decrease. The amount of decrease depended upon the size of the muscle with smaller muscles exhibiting better tension recovery and less net K^+ efflux.

The application of high pCO_2 to resting muscle was also found to cause an increase in the rate of net K^+ efflux and a corresponding decrease in twitch tension. Both of these observations may be explained on the basis of a net $H^+ - K^+$ exchange in which K^+ ions are displaced by H^+ ions within the cell both during fatigue and during intracellular acidosis produced by high pCO_2. During the recovery phase H^+ ions are pumped out of the cell into the extracellular fluid where they are buffered while K^+ ions return to the interior of the cell regaining their sites on the fixed anionic charges temporarily held by the H^+ ions.

The possibility of using high pCO_2 to simulate muscle fatigue is discussed on the assumption that intracellular acidosis plays a major role in the development of muscle fatigue.

CHAPTER I

Introduction

1. Membrane theory

a) Development of the membrane theory

In 1890, Ostwald studying the electrical potentials generated across semipermeable precipitation membranes suggested that cellular electrical potentials could be explained in terms of the characteristics of such a membrane. Approximately twelve years later McDonald (1900, 1902) and Bernstein (1902) independently developed this idea into the theory of membrane potentials. These investigators reasoned that, if the cell membrane did not allow the passage of negatively charged anions but permitted some cations to pass through, the cellular electrical potential would arise from this semipermeability of the cell membrane. Since the major cation found inside muscle and nerve cells is potassium, the cell membrane must be permeable to this ion; and the potential established should obey the Nernst equation:

$$E_m = \frac{RT}{F} \times 1_n \frac{K_i}{K_o}$$

It was further postulated that this selectivity for potassium was lost during excitation at which time other small ions were allowed to enter the cell through numerous expanding pores in the cell membrane. This theory remained unchallenged until 1939, when Hodgkin and Huxley showed that excitation leads to a transient reversal, not a simple abolition, of the resting potential. Boyle and Conway (1941) reported that the

skeletal muscle fiber is permeable to chloride as well as to potassium and that its osmotic and electrochemical behaviour could be explained by the thermodynamic treatment of the equilibrium state in a two-phase system separated by a semipermeable membrane as described by Donnan in 1911. Therefore, the muscle fiber seemed to follow the rules of a Donnan equilibrium, its surface membrane being, in effect, impermeable to the large organic anions synthesized inside the cell and to the external sodium ions with the permeant ions (mostly K^+ and Cl^-) distributing themselves such that:

$$\frac{K_i}{K_o} = \frac{Cl_o}{Cl_i}$$

Contrary to these earlier conclusions it was shown that the membrane is permeable to Na^+ (Heppel and Schmidt, 1938, Heppel, 1940; Levi and Ussing, 1948; Harris and Burn, 1949; Steinback, 1951; Keynes, 1954).

$$E_m = \frac{RT}{F} \times 1_n \frac{K_i + Na_i}{K_o + Na_o}$$

However since the sum K_i plus Na_i is approximately equal to the sum K_o plus Na_o, this equation predicts no potential across the membrane whatever. The problem was approached by Hodgkin and Katz (1949 b) who adopted Goldman's (1943) modified constant field equation which includes the permeability of the Na^+:

$$E_m = \frac{RT}{F} \times 1_n \frac{P_K K_i + P_{Na} Na_i + P_{Cl} Cl_o}{P_K K_o + P_{Na} Na_o + P_{Cl} Cl_i}$$

(Where P_K, P_{Na} and P_{Cl} represent permeability constants.) In the case of frog skeletal muscle, the ratio between these ion permeabilities is P_K: P_{Cl}:P_{Na} = 1 : 2 : 0.01 while in Loggio giant axion it is P_K:P_{Cl}:P_{Na} = 1 : 0.45 : 0.04 (Hodgkin, 1951). Since in the resting state $P_{Na} << P_K$ the problem of Na^+ permeability on the magnitude of the resting potential is resolved. However, the concept on which the above equation is based does not directly suggest a mechanism for the maintenance of the low intracellular Na^+ concentration found in resting cells. This function has thus been delegated to a hypothetical sodium pump energized by metabolic reactions. (Hodgkin, 1951).

b) Structure of the membrane

Overton, in 1899 postulated that the plasma membrane was a film of fat-like substances which he termed lipoids. However, this theory was abandoned because of its inability to explain the permeability of cells for water, urea, and many other substances which are capable of penetrating cells very quickly but are completely insoluble in lipoids. Nathanson (1904) tried to explain the permeation of substances insoluble in lipoids by suggesting that the membrane consisted of cholesterol on the surface of the cell between large particles of protoplasm giving the membrane a mosaic structure. However, since this theory had no experimental foundation it was quickly abandoned. Ruhland in a series of articles (1908 - 1913) put forward the theory of the ultra-filter stating that the penetration of a substance into cells depends upon its molecular volume and therefore the semipermeable membrane worked on a seive principle. This seive theory could not however explain the familiar fact

that the permeability of many plant and animal cells for substances of homologous series increases with the increase of the chain of the number of carbon atoms as shown by Collander and Barlund (1933).

Therefore the theory was extended to a combined lipid theory and molecular seive theory. According to this lipid filtration theory the permeation of a substance insoluble in lipids is regulated by the size of the pores in the membrane, while that of the substances soluble in lipids is regulated by the coefficient of the distribution of the substances between the non-aqueous and aqueous phases.

Danielli (1935, 1936) and also Harvey and Danielli (1939) proposed that the protoplasmic membrane should consist of two orientated layers of lipoid molecules whose hydrophillic ends point in opposite directions (to the inside and to the outside of the cell). The molecules of the orientated layers of the membrane are on the surface of the cell in the form of a palisade. Because the surface-tension of the protoplasm is very low it is supposed that the membrane is covered on both sides with a layer of globular proteins which greatly reduce the surface tension of the cell. At present a large number of schemes have been suggested for the structure of the cell membrane, however, most of these are based on a lipid protein combination as proposed by Davson and Danielli.

c) Movement of substances across the membrane

As outlined earlier, the cell membrane probably consists of outer and inner monomolecular layers of protein separated by a bimolecular lipid layer. In view of this probable membrane structure the problem becomes not one of accounting for the low permeability of the membrane to

water soluble substances but explaining the occurance of any penetration at all. Lipid-soluble substances presumably penetrate by dissolving in the membrane substance. Other substances must thus traverse the membrane in one of the following ways.

i) Pore theory -

Boyle and Conway (1941) suggested that some ions such as K^+, Rb^+, Cl^-, Br^- and NO_3^- freely pass into muscle fibers while others such as Na^+, Li^+ and $SO_4^=$ do not due to the size of their respective hydrated diameters. They suggested that, if the hydrated diameter of the ion were larger than the mean pore diameter of the membrane, the passage of the ion through the aqueous phase of the pore would be impeded. Barring one or two exceptions, Conway found that ions with diameters less than a certain critical value were permeant, while those with greater diameters were impereant. However, this theory failed to explain why some ions such as Cs^+ and Rb^+ which have hydrated diameters identical to or very equal to K^+ failed to diffuse into the muscle at the same rate as K^+.

In order to explain this discrepancy Conway (1960) suggested that the number of water molecules bound in hydration might also influence permeability if those with many shells allow a certain degree of deformation, thus facilitating passage through a pore.

An alternate explanation for the apparent discrepancy between hydrated ion radius and permeability is provided by the solvation hypothesis of Mullins (1956). According to this theory ions in solution will penetrate the membrane with pore radii of less than 10 Å only if the ion and pore radii are similar. Mullins suggests that not all the pores in the

membrane are the same size but that the mean radius is 4.05 Å. The Na^+ and Li^+ ions are too large to pass through such pores but smaller ions can replace hydration beyond the first shell with solvation of similar dimension obtained from the wall of the pores. Thus, the hydrated ion may only pass through the pore if its radius is identical with that of the pore, otherwise there will be a space around the ion where the dielectric constant will be 1 or where the charge will be unshielded. To avoid the all-or-none element introduced by these assumptions Mullins visualizes a hypothetical distribution of the pores with respect to size in a Gaussian curve. This dispersion in pore radius also provides an explanation for the increased permeability to sodium during excitation. Namely, that at rest the Gaussian distribution of pores is such that it favours the passage of K^+ ions, while depolorization of the membrane leads to a redistribution of the pores with a mode at the size of the Na^+. Finally there exists the possibility that fixed charges on a membrane may also influence ion permeability. This was first suggested by Michaelis (1925) on the basis of observations made with charged collodion membranes and developed simultaneously by Teorell (1935, 1937) and K.H. Meyer and Scivers (1936). These investigators believe that the walls of the pores may bear electrical charges located on the side groups of protein molecules or on phosphate groups bound to these molecules. The positively charged pores would allow the passage of anions while the negatively charged pores would allow only the passage of cations. The potential difference created by the charged membrane would be superimposed on the diffusion potential since the value of the latter depends on whether the cations or the anion

has the greater mobility. Thus, one could obtain not only all degrees of diminution of the diffusion potential but an actual reversal of its sign. More recent elaborations on this theory have been carried out by Teorell (1959) and Manery (1966).

ii) Carrier hypothesis -

According to the carrier hypothesis, there is a special lipid-soluble carrier molecule (possibly a phophatide) within the membrane. This carrier molecule is capable of combining preferntially with a particular ion or substance. An ion from the interstitial fluid could combine with this carrier molecule at the surface of the membrane; the ion-carrier complex might then diffuse through the membrane to the inner surface where the ion would dissociate from the carrier and enter the intracellular fluid.

The carrier mechanism may operate passively down a concentration gradient not requiring metabolic energy. This type of "facilitated diffusion" has been used to describe the passage of sugar into frog muscle (Narahara and Ozand, 1963).

The situation for the removal of Na^+ from the cell is quite different. Since the direction of the chemical gradient for Na^+ is inward and the electrical gradient is in the same direction, some form of metabolic energy must be expended to work against this electrochemical gradient, i.e. active transport.

iii) Active sodium transport -

Hodgkin and Keynes (1955) have demonstrated that removal of K^+ from the bathing medium reduced the Na^+ efflux from the axon of Sepia to

about 0.3 of its previous value. Harris and Maizels (1951), and Glyn (1956) studying Na^+ efflux in human red blood cells found that the pump output of Na^+ was completely inhibited in the absence of external K^+. These findings suggest that there is an active uptake of K^+ coupled with Na^+ extrusion. A coupled Na^+ - K^+ exchange mechanism is also suggested by the observation that a decrease in the amount of available energy either by metabolic inhibitors or by temperature reduction has paralleled effects in reducing Na^+ efflux and K^+ influx (Hodgkin and Keynes, 1955).

Although K^+ appears to be actively transported in erythrocytes (Post and Jolly, 1957; Gill and Solomon, 1959) a number of investigators (Page and Storm, 1956; Adrian and Slayman, 1966; Ritchie, 1967) have proposed an electrogenic Na^+ - K^+ exchange mechanism in nerve and muscle. Thus, K^+ would move passively into the cell under the influence of a potential generated by the activity of the pump. As yet there is no conclusive evidence for either the "concentration" or "electrogenic" nature of the pump suggesting possibly a mechanism that contains some of the features of each.

Within the last ten years a number of important discoveries have aided in the understanding of the molecular basis of Na^+ - K^+ transport. This work has been carried out both in human erythrocytes (Post, et al., 1960; Dunham and Glyn, 1961) and nerves of the squid (Keynes, 1961) and crab (Skou, 1957). The findings of the above investigators and those of many others may be briefly summarized as follows: a) adenosine triphosphate (ATP) is an energy source sufficient to maintain pumping in squid nerve and in red cell ghosts; b) there exists in the membrane of these

structures a form of ATPase which is activated by the presence of Na^+ and K^+; c) this enzyme has a mandatory requirement for Mg^{++} and is inhibited by Ca^{++}; d) this ATPase activity is also inhibited by cardiac glycosides such as Ouabain; e) competitive inhibition of Na^+ activation by K^+ and of K^+ activation by Na^+ has been observed. However, a valid model containing the above features relating the enzymatic processes to the chemical and mechanical processes of active ion transport remains a challenge.

2. Bulk-phase theory

The membrane theory of cell permeability is perhaps the most widely held; but it has been challenged on many occasions by those who believe that selective permeability is determined by the physical chemical properties of the bulk-phase of the cell rather than a limiting membrane. They do not deny the existence of a distinct outer region of the protoplasm which is about 70 Å in thickness and easily distinguishable under the electron microscope but they do not consider that this structure has any significance in cell permeability. There exist today two main schools of thought as to the nature of the bulk-phase; viz., the sorption theory of Troshin and the fixed charge-induction hypothesis of Ling.

a) Sorption theory of Troshin

The sorption theory of Troshin is patterned after a theory of excitation based on a denaturation-like protein configuational change expounded of D.N. Nassonov (Nassonov and Aleksondrov, 1940; Nassonov, 1959). Troshin (1958) worked out a complete development of this theory

and applied it to his experimental data and to those published in the literature. This theory is called the sorption theory of cell permeability because sorption factors such as solubility, adsorption and chemical binding of substances in protoplasm play a major role in it. According to the theory selective sorption by the protoplasm of a given substance is due to the peculiarities of the structure of the protoplasm itself which in turn is maintained by the energy of metabolism. All water present in the protoplasm is in the organized or "bound" form and therefore the solubility of substances in it is lower than in ordinary water. The higher concentration of some substances inside the cell as compared with the medium is explained by the postulate that these substances also exist in protoplasm mostly in a bound state due to differences in the readiness of dissolving and fixation in protoplasm between substances.

Troshin (1960) reported that the viscosity of cytoplasm changed during excitation and that the isolated proteins from stimulated cells had lower affinity for K^+ and a higher affinity for Na^+. This may be explained by the protein denaturation theory of excitation and injury which Nassonov developed as mentioned earlier. Basically, excitation of cells is associated with an alteration of protoplasmic proteins resembling the denaturation of native proteins. As a result of this alteration of proteins the complex colloid structure of the protoplasm is disturbed causing an increase in the solubility of substances in the protoplasm. Such altered cell proteins change abruptly their capacity to bind (chemically and by adsorption) mineral or organic ions present in the cell or taken up by it leading to a redistribution of substances between the cell

and the medium.

b) Fixed-charge induction hypothesis of Ling

In 1951 and 1952 Ling published the original version of his fixed-charge hypothesis in which he suggested that the densely packed cellular proteins are highly organized as shown by the X-ray diffraction pattern of muscle cells (Astbury, 1939). The hypothesis assumed that the high density of charges carried by the trifunctional amino acids are fixed in space and that association between oppositely charged ions becomes tremendously increased if one species of the ion is fixed in a macroscopic three-dimensional latticework. Thus, selective ionic accumulation follows from a difference in the electrostatic energy between the fixed charges and the interacting ions. Ling assumed at this time that the fixed anionic charges in living cells were predominantly carboxyl groups carried by aspartic and glutamic residues of the proteins and that they are unvarying. It followed that the electrostatic interaction energy would then be determined by the average dielectric constant and by the "distance of closest approach" between the fixed anion and cation. A demonstration of selectivity in K^+ accumulation over Na^+ was possible assuming that the "distance of closest approach" was determined by the hydrated radii of the competing cations.

However, it soon became clear that the model was incapable of explaining an increasing amount of evidence for the existence of alkali metal ion adsorption in sequences other than that of the hydrated diameters and that variation of the "anionic field strength" leads to "particular" order of ionic selectivity (Eisenman, Rudin and Casby, 1957;

Rudin and Eisenman, 1959). Because of this a new microscopic model (1957) was developed with all known physical forces including Coulomb's and van der Wall's and other energies taken into account in a calculation of the adsorption energies of the various cations. In the revised form of the hypothesis instead of presenting as an assumption the assertion that fixation increases the association of a charge's counterion, an explicit mechanism is now given in terms of statistical mechanics. The predicted increase of ionic association with fixation of one species of ion provides some possible answers to the lack of a theory for potential differences between two ionic phases and the failure to demonstrate selective ionic adsorption in isolated protein solutions. Also, neither the fixed ions nor their counterions are regarded as unvarying. Instead, the fixed protein carboxyl groups are allowed to vary what may be loosely called their effective charge or more rigorously defined by Ling as their "c" value. The cations are not assumed to have any definite hydrated diameter, rather a statistically varying number of water molecules are interspersed between the fixed anions and the free cation and these are permitted to vary with both the "c" value of the anion and the nature of the cation investigated. The "c" value of the anion will vary in response to changes on the protein molecule at some distance. It is proposed (Ling, 1957; 1958) that such a long range interaction is made possible by the high polarizability of the resonating polypeptide chain "backbone" and through the combined action of the induction and the direct electrostatic effects, termed the "F" effect, of the absorbed counterions. An important variation of this direct "F" effect, called an indirect "F"

effect is visualized to enable protein molecules to propagate an electronic charge to points beyond those reached by the direct induction and direct electrostatic effects. Because the fixed charge hypothesis is no longer limited to dealing with static problems but, by the induction effect, describes also changes in the fixed charge system the theory is now termed the fixed charge-induction hypothesis.

c) Three phase theory

To help explain the results of radioactive isotope exchange studies, a number of investigators have postulated that perhaps not all intracellular potassium is bound but, perhaps there exists within the cell a small portion of freely exchangeable potassium. One of the foremost proponents of this idea is S.E. Simon and her associates (Simon, et al., 1957, 1959; Tasker, et al., 1960; Simon, 1960) who have proposed a three-phase theory of transport.

The three phases postulated are the extracellular phase, the free intracellular phase and the ordered phase. It is assumed that those ions which are accumulated by the cell are adsorbed by the ultrastructure of the ordered phase, which is envisaged as a highly ordered macromolecular gel extending throughout the greater part of the cell. The free intracellular phase has been defined as an intracellular region intermediate in order between the extracellular phase and the ordered phase. It would seem probable that this phase could be identified with the sarcoplasmic reticulum.

d) Pitfalls of the bulk-phase theory

The inability of some of the sodium and potassium of tissues to be extracted by solutions free of these ions, or to exchange with external tracer ions, has been interpreted as independent evidence for the binding of intracellular cations. Both Ling (1952) and Shaw and Simon (1955) claimed that most of the potassium normally present in frog muscles was bound to intracellular structures because it was retained against a concentration gradient after the muscles had been poisoned to abolish metabolic sources of energy. Troshin (1957) reported that a fifth of the sodium in frog muscles exchanged very slowly, and an earlier suggestion has been revived (Bozler, et al., 1958 and Bozler and Lavine, 1958) that a large part of the cations in smooth (gastric) muscle of the frog was held by adsorption rather than by a selectively permeable membrane, and that osmotic balance was only maintained because a large part of the water was also "bound". However, Harris (1961) and Dydynska and Wilkie (1963) have demonstrated that all the water in muscle fibers is free to participate in osmotic activity when immersed in hypotonic or hypertonic solutions.

According to the bulk-phase theory at least 80% of cell K^+ is bound and therefore its activity coefficient within the cell should be lower than that for K^+ in free aqueous solution and closer to the activity coefficient of K^+ in an ion exchange resin. Gregor and Gottlieb (1953) found the activity coefficient of K^+ in an ion exchange resin which could be compared with the Ling cell model to be as low as 0.39. Hinke (1961) using cation-selective glass microelectrodes measured the

activity of K^+ and Na^+ in squid axon and in extruded axoplasm and found it to be 0.605. Since a 0.1 M solution of KCl in water has an activity coefficient of 0.77 (Conway, 1952) Hinkė's result would suggest that at least 88% of the K^+ in the axoplasm was in free solution. Lev (1964) found activity coefficients between 0.751 and 0.795 also employing glass microelectrodes but in frog muscle fibers. Furthermore, he observed that the activity coefficient for Na^+ in the same preparation was 0.200 indicating appreciable binding of this ion. This is a direct contradiction to the prediction of the bulk phase-theory in which K^+ binding should be greater than Na^+ binding in the cell.

Ling (1969) holds that the piercing of the cell with a microelectrode undoubtedly creates profound disturbances in the region immediately surrounding the electrode. Thus, the K^+ ion activity measured with an intracellular electrode would not be expected to reflect the activity of the K^+ ion in its normal resting state.

The results of Hinke (1961) and Lev (1964) substantiate earlier work on the mobility of ^{42}K under the influence of an electric field in nerve axon (Hodgkin and Keynes, 1953) and muscle fiber (Harris, 1954) which also suggested that K^+ is in free solution within the cell. Ungar (1963) believes that these electric field studies are compatible with the ion being adsorbed on an ion exchange system in which the counterion can move along a series of charged sites without ceasing to be bound.

However, neither myosin nor actomyosin exhibit a selective binding of K^+ as one would expect from the bulk phase theory (Erdos, 1946).

Although in the light of more recent work (Lewis and Saroff, 1957) myosin may be an important exception. Also, organic phosphates and other cell constituents seem to show no significant preference for K^+ over Na^+ (Car, 1956; Ussing, 1960).

3. Factors which effect K^+ flux in muscle - Membrane theory

Ion fluxes in a resting muscle may be represented as shown in Fig. 1. Here we see that the net potassium efflux is equal to the passive efflux of K^+ minus the passive influx.

$$\begin{aligned} \text{Net passive efflux} &= \text{passive efflux} - \text{passive influx} \\ &\quad (5.4 \text{ pM cm}^2 \text{ sec} - 3.6 \text{ pM cm}^2 \text{ sec}) \\ &= 1.8 \text{ pM cm}^2 \text{ sec} \end{aligned}$$

This net passive efflux of potassium is just sufficient to balance the passive influx of Na^+.

If we assume that there is a one-for-one $Na^+ - K^+$ exchange due to the sodium pump then, in the steady state, this net passive efflux of K^+ is balanced by the active K^+ influx (1.8 pM cm^2 sec) of the pump. Thus, in the resting steady state, the net fluxes of K^+ and Na^+ across the cell membrane should be zero.

However, when a muscle is isolated from an animal and placed in normal Ringer's solution it tends to lose K^+ in exchange for Na^+. This net entrance of Na^+ would be expected to depress the membrane potential. In order to maintain ionic equilibrium in an isolated muscle it is necessary to increase the external K^+ concentration of the bathing

Fig. 1

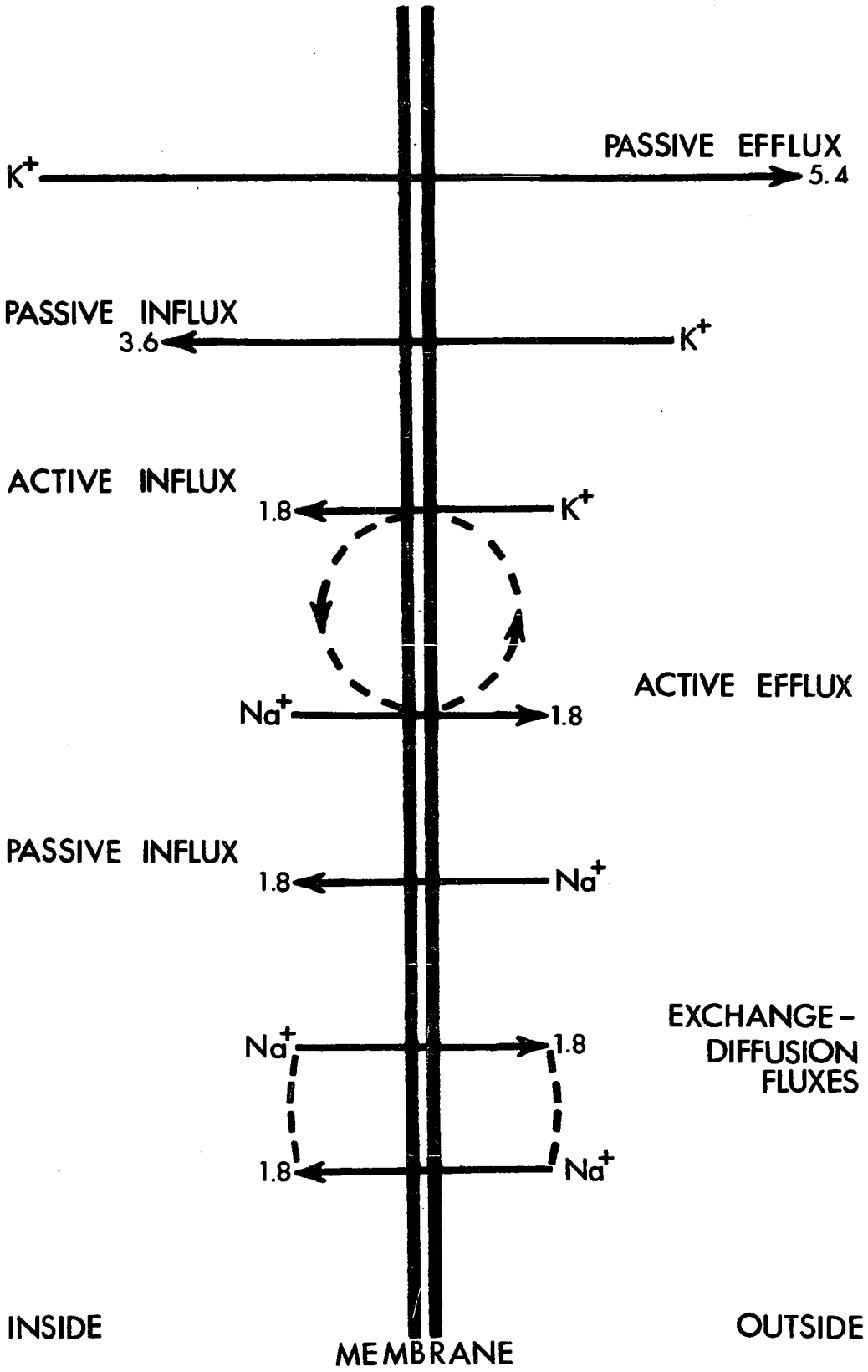
Potassium fluxes in frog sartorious muscle.

All flux values are in pmols/cm^2/sec.

References: a) Flux data - i) Hodgkin and Horowicz (1959)

ii) Keynes and Swan (1959)

b) Diagram after Swan (1961).


PASSIVE EFFLUX
K^+
5.4
PASSIVE INFLUX
3.6
K^+
ACTIVE INFLUX
1.8
K^+
ACTIVE EFFLUX
Na^+
1.8
PASSIVE INFLUX
1.8
Na^+
EXCHANGE-DIFFUSION FLUXES
Na^+
1.8
1.8
Na^+
INSIDE
MEMBRANE
OUTSIDE

solution. The magnitude of this increase depends on both the temperature (Boyle and Conway, 1941) and the pH of the solution. Fenn and Cobb (1934) found that an external K^+ concentration of 4.8 mM was required to maintain K^+ equilibrium in frog sartorious muscles in a bathing solution having a pH of 7.2. When the pH of the solution was decreased to 5.6 (by altering its $NaHCO_3$ content) the K^+ concentration of the bathing solution had to be increased to 11.8 mM to stop net loss of K^+ from the muscle. This tendency for K^+ to exchange for H^+ will be discussed in greater detail later. Of interest here is the fact that as in vivo conditions are approached this K^+ maintenance concentration is found to decrease. Mond and Amson (1928) reported that a perfusing solution containing 3.3 mM K^+ was sufficient to maintain K^+ equilibrium in the perfused hind limb of the frog.

As mentioned earlier Na^+ enters isolated frog muscle in exchange for K^+ depressing the membrane potential. Carey and Conway (1954) and Creese, D'Silva and Northover (1958) observed that when isolated muscles were bathed in plasma or serum they took up less Na^+ than those immersed in Ringer. It has also been shown that above the maintenance concentration, the Nernst equation describing the K^+ equilibrium potential holds quite well for muscle (Conway, 1957; Adrian, 1956). Kernan (1960) found the membrane potential of frog sartorious muscle fibers immersed in plasma having a K^+ concentration near 2.5 mM to be 99.2 ± 0.7 mv compared with a calculated value of 100.1 mv. Likewise the same investigator (1963) found a discrepancy of less than 1 mv between the observed and calculated membrane potential in rat extensor digitorum muscle

TABLE I

Concentrations of various ions
in
isolated frog sartorious muscle

CONCENTRATIONS

	Inside	Outside
K^+	140.0 mM	2.5 mM
Cl^-	3.6 mM	120.0 mM
Na^+	9.2 mM	120.0 mM
A^-	152.0 mE	

POTENTIALS

E_m = resting membrane = -94 mv.

$$E_K = \frac{RT}{F} \ln \frac{(K)_o}{(K)_i} = -101 \text{ mv.}$$

$$E_{Cl} = \frac{RT}{F} \ln \frac{(Cl)_i}{(Cl)_o} = -88 \text{ mv.}$$

$$E_{Na} = \frac{RT}{F} \ln \frac{(Na)_o}{(Na)_i} = +64 \text{ mv.}$$

Where E = internal potential
F = Faraday's constant
R = the gas constant
T = the absolute temperature

bathed in oxygenated plasma having a K^+ concentration of 5 mM. Moor and Cole (1960) measuring the resting potential in the giant nerve axon of the squid found it (77mv) to be close to the K^+ equilibrium potential.

Thus, it appears that the membrane potential of muscle in the resting steady state is a K^+ diffusion potential and the discrepancy between the K^+ equilibrium potential and the resting membrane potential measured in isolated muscles (see Table I) immersed in normal Ringer's is due to the absence of a balanced state where the net flux of K^+, Na^+ and Cl^- is zero.

On the basis of the previous discussion the following factors will affect K^+ flux:

a) Changes in the net indiffusable internal charge either by loss of indiffusable anions (A^-) from the cell or by neutralization of their charge.

b) Changes in the membrane potential thus altering the permeability of the membrane to K^+ or Na^+.

c) Changes in the ability of the active transport mechanism to balance K^+ loss or Na^+ gain.

a) Changes in the net indiffusable internal charges

According to the membrane theory the high internal K^+ concentration is due to the fact that the cell membrane does not allow the passage of negatively charged anions out of the cell and due to its selective permeability to K^+ allows this ion to enter the cell and to neutralize

the negatively charged anions. Thus, the fixed negative charge of the cell interior due to the stability of the anionic macromolecules is an important factor in the maintenance of a high intracellular K^+ concentration. Consequently, any change in this anionic concentration may be expected to alter K^+ distribution across the cell membrane. It has been observed that removal of mammalian muscle results in a rapid increase in the total molecular concentration within the cell, as measured cryoscopically, suggesting hydrolysis of large molecules (Conway, et al., 1955). If diffusable anionic molecules were produced, a loss in cell K^+ could occur due to a shift in the Donnan and other equilibria. Baetjar (1935) found that the potassium concentration in mammalian tissue seems to be dependent upon respiration which may be needed for the structural integrity of the cell. Mullins and Noda (1963) have demonstrated that frog muscle fibers may lose as much as 0.68 meq/Kg/hr of phosphate in the absence of external chloride and that this loss is paralleled by a loss in potassium.

Neutralization of the total anionic charge within the cell itself would also lead to increased K^+ efflux. For example, when a muscle contracts K^+ ions leave the cells and H^+ ions formed as a result of the metabolic contracting process may replace the sites on the anions previously occupied by the K^+ ions.

$$A^- + H^+ = AH$$

Before K^+ ions can regain their previously held anionic sites the excess H^+ ions must be eliminated. This depends, of course, on the cell's

buffering capacity and its ability to regulate intracellular pH. Therefore, since these large anionic macromolecules play a role in intracellular buffering, it follows that changes in intracellular pH would exert an effect on K^+ equilibrium. Because variations in intracellular pH are capable of altering K^+ flux, we shall briefly discuss its measurement, possible modes of regulation and the changes in K^+ flux which occur in response to changes in intracellular pH.

The values obtained for intracellular pH are as wide and as varied as the methods of measurement. These methods include measurement of the pH of homogenized tissue or an extract of tissue fluid withdrawn from the cell; measurements employing coloured pH indicators both natural and artificial; measurement of a weak acid or base and its corresponding ion content in the cell and finally determinations with pH sensitive glass microelectrodes. Of all these only the last two have gained popular appeal and therefore these methods will be discussed in detail. Two substances have been employed in the weak acid or base method. These are carbon dioxide and the weak organic acid 5,5-dimethyl-2,4-oazolidinedione or DMO.

i) Measurement of intracellular pH -

The carbon dioxide method was used by Fenn (1928), Stella (1929) and Ferguson (1929) to determine the pH of frog muscle and nerve. The total carbon dioxide content of the tissue is determined by treating it with acid. If we assume that the intracellular partial pressure of carbon dioxide is the same as that of the surroundings and that the total amount of carbon dioxide present is in the form of HCO_3^- and dissolved

CO_2 then the intracellular pH may be calculated from the Henderson-Hasselbach equation:

$$pH = pK - \log_{10}\frac{(CO_2)}{(HCO_3^-)}$$

where (CO_2) represents the combined concentration of H_2CO_3 and dissolved CO_2 and the pK value is that of the serum. With this method Fenn and Maurer (1935) obtained a vlaue of 6.9 for the pH of frog muscle and Stella (1929) a value of 6.98.

This method was questioned by Conway and Fearon (1944) who demonstrated that in the case of rat, rabbit, cat and guinea pig muscle not all the carbon dioxide liberated comes from bicarbonate but some comes from a substance which does not appear to be a carbamino compound. Making allowances for this compound these investigators calculated the pH of rabbit muscle to be 6.0. This value also supported Conway and Fearon's view that the distribution of H^+ ions is obeyed by a Donnan ratio. However, the objection to the use of CO_2 because of the existence of a barium-soluble fraction has since been shown to be unfounded (Butler, et al., 1967).

In 1959, Waddell and Butler found that the weak organic acid DMO possessed the physical and chemical attributes of a suitable indicator of intracellular pH. The ionization exponent of DMO is almost identical to the apparent first ionization exponent of carbonic acid thus making it as sensitive an indicator as is theoretically possible for an organic acid. DMO does not bind to plasma protein or intracellular constituents, it does not enter fat, has low toxicity, is

not metabolized and is excreted very slowly by the kidney. Since it is not metabolized and is nonvolatile, the radioactively labelled compound can be used and determined by simple specific methods of great sensitivity. If it is assumed that the concentration of unionized DMO in the cell water is equal to the concentration of unionized DMO outside the cell and that pK of DMO in and out of the cell are identical, then the intracellular pH may be calculated from the distribution of DMO between the muscle and the plasma.

Waddell and Butler (1959) using DMO found the intracellular pH of dog skeletal muscle to be 7.04 and Irvine (1961) obtained a value of 6.94 for the intracellular pH of rat gluteal muscle. Adler, et al. (1965) employing ^{14}C-labelled DMO found that the intracellular pH of rat diaphragm to be 6.90 at an external pH of 7.38; while Miller, Tyson and Relman (1963) also employing ^{14}C-labelled DMO obtained a value of 6.94 at an external pH of 7.38 for rat diaphragn.

It should be noted in passing that in the study of Miller, Tyson and Relman both the DMO and the CO_2 methods were compared in the same rat diaphragm preparation with quite close agreement at high values of pCO_2. The carbon dioxide method did however, yield higher pH values than the DMO method at very low values of pCO_2.

A third method of determination consists of inserting a H^+ sensitive glass microelectrode into the cell with a reference electrode in the bathing solution. Caldwell (1958) employing capillary electrodes with a tip diameter of 50 - 100 microns measured the intracellular pH of crab muscle fibers and obtained a value of 7.15. With a similar tech-

TABLE II

Values of intracellular pH according to method of measurement

The Carbon Dioxide Method

Investigators	Muscle	pH
Fenn and Maurer (1935)	frog	6.90
Conway and Fearon (1944)	rabbit	6.00
Stella (1929)	frog	6.98
Miller, et al. (1963)	rat	6.86
Tobin (1956) (1956)	cat	7.28
Benson, et al. (1965)	dog	7.13

The DMO Method

Investigators	Muscle	pH
Waddell and Butler (1959)	dog	7.04
Irvine et al. (1961)	rat	6.94
Adler, et al. (1963)	rat	6.90
Miller, et al. (1963)	rat	6.94
Brown and Goott (1963)	dog	6.93
Schloerb (1963)	dog	6.84
Brown, et al. (1967)	dog	6.95
Miyao (1967)	rat	7.03

The Glass Microelectrode Method

Investigators	Muscle	pH
Caldwell (1958)	crab	7.15
Kostyuk and Sorokina (1961)	frog	7.12
Hinke and McLaughlin (1967)	barnacle	7.43
Carter, et al. (1967)	rat	5.77-7.14

nique Kostyuk and Sorokina (1961) observed the intracellular pH of frog sartorious muscle to be 7.1. More recently Hinke and McLaughlin (1967) found the intracellular pH of barnacle muscle to be 7.43 at external bath pH of 7.54. In opposition to these results, Carter (1967) using single, double and triple-barreled microelectrodes has determined the intracellular pH of rat skeletal muscle to be in the range of 5.77 - 6.29. Carter contends that Caldwell's electrodes may have caused alkalinizing injury of the cell cytoplasm due to their size while those of Kostyuk and Sorokina were poorly insulated with shellac. The results of the various methods are tabulated in Table II.

ii) The regulation of intracellular pH -

There are two possibilities which exist for the regulation of intracellular pH. First that regulation is a purely passive thing governed by the Donnan equilibrium or second, that the regulation of intracellular pH involves the active transport of H^+ ions at the expense of metabolic energy.

An equilbrium of the Donnan type arises when there exists a boundary between solutions of ions and one or more of the ionic species is unable to cross the boundary. If K^+ and H^+ ions obeyed this type of equilibrium then the following relationship would hold:

$$\frac{(H^+ \text{ extracellular})}{(H^+ \text{ intracellular})} = \frac{(K^+ \text{ extracellular})}{(K^+ \text{ intracellular})}$$

and the Donnan equilibrium theory relationship for pH across a membrane at 20°C would be:

$$\text{Internal pH-pH of surroundings} = \log_{10} \frac{(H^+ \text{ Activity in surroundings})}{(\text{internal } H^+ \text{ Activity})}$$

$$= \frac{\text{Resting potential (mv)}}{58.17}$$

In 1935, Fenn and Maurer found the pH of the extracellular fluid in frog muscle to be about 7.4. Calculation of the intracellular pH either from the intracellular and extracellular potassium concentrations (first equation above) or from the normal resting potential of about -90 millivolts (second equation above) shows that it should be below 6.0. However, a glance at Table II indicates that most of the experimental values obtained are in the region of 7.00. Caldwell (1954, 1956) has likewise demonstrated that the value of the intracellular pH of crab muscle as predicted by the Donnan equilibrium (pH 6.7 -6.4) is in contrast with his experimental findings (pH 6.9 - 7.15). Hill (1955) employing a different approach based on the production of lactic acid has shown that in normally excitable muscle the Donnan equilibrium does not control, or greatly influence the distribution of H^+ ions across the fiber membrane. Conway (1957) however, feels that although the electrolyte equilibrium in muscle may not be a simple Donnan equilibrium acute changes in pH result in net diffusions towards the attainment of such a relation. This belief is also held by Fenn (1958).

A number of models have been proposed to explain active H^+ transport across the cell membrane, two of which are illustrated in Fig.2. The first (A) consists of a simple H^+ pump with an accompanying leak of HCO_3^-. The second model (B) maintains that H^+ is capable of combining

Fig. 2

Possible models for transmembrane H^+ transfer.

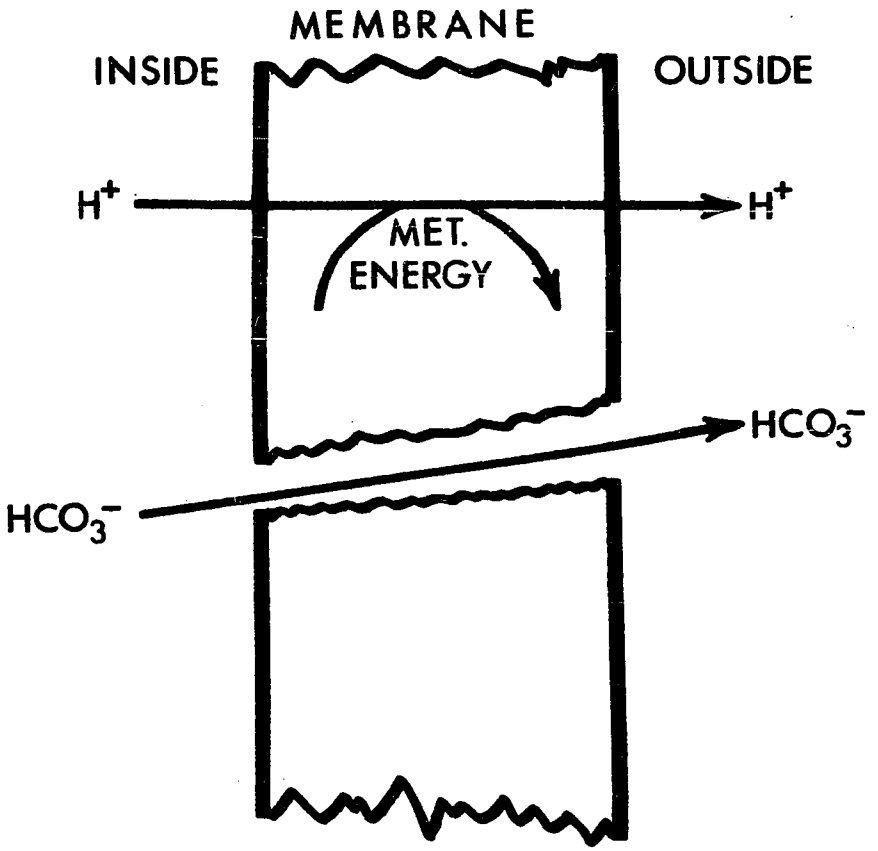
Fig. 2 a

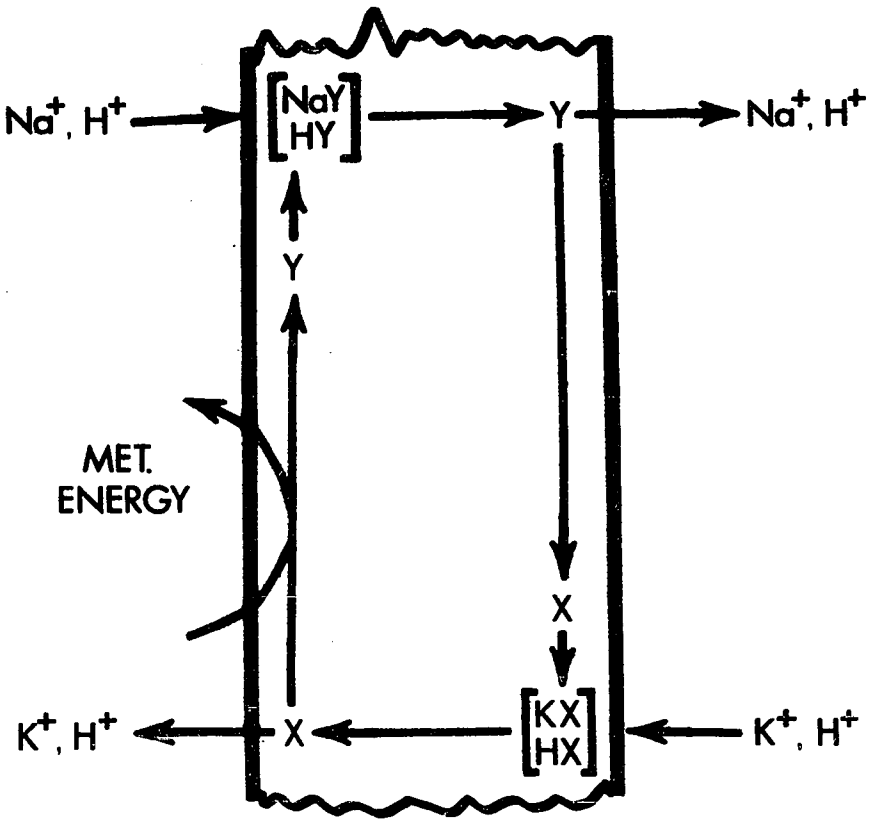
Simple model of H^+ pump with accompanying leak of HCO_3^-.

Fig. 2 b

Two-way H^+ transport via the coupled Na^+-K^+ pump.

Reference: Ruch and Patton, 1965


MEMBRANE
INSIDE
OUTSIDE
H+
H+
MET.
ENERGY
HCO3−
HCO3−


Na+, H+
[NaY
HY]
Y
Na+, H+
Y
MET.
ENERGY
X
K+, H+
X
[KX
HX]
K+, H+

with the same carriers used by Na^+ and K^+ in the coupled $Na^+ - K^+$ pump. This provides for two-way H^+ transport, H^+ is pumped out of the cell by combining with the Y carrier of Na^+ and into the cell by combining with K^+'s X carrier. The affinity of H^+ for the Na^+ carrier Y, must be greater than the affinity of H^+ for the K^+ carrier X in order to maintain a low internal H^+ concentration.

Davies and Keynes (1961) have proposed another variation of the $Na^+ - K^+$ pump which is driven by a $H^+ - OH^-$ pump in which H^+ ions combine with the X and Y molecules on their return trips through the membrane.

iii) The response of K^+ flux to alterations in intracellular pH -

Fenn and Cobb (1934) reported that potassium moved out of frog skeletal muscle into the bathing solution when the muscle was immersed in saline and pH was lowered by increasing CO_2 tension of the gas in equilibrium with the system. When the experiment was repeated with blood as the bathing fluid instead of saline, K^+ was found to move in the opposite direction (Fenn and Cobb, 1935). In 1950, Fenn and Gerschman obtained the same results with frog nerves. The explanation for these results as given by Fenn (1928, 1956) was that in the first case (1934) tissue was a better buffer than saline and in the second (1935), since the bicarbonate content of whole blood is about twice that of muscle and since the slope of the CO_2 dissociation curve is steeper in blood than in muscle it can be predicted that CO_2 will cause a greater fall in pH in muscle than in whole blood. Hence, CO_2 should drive K^+ from plasma into muscle, the basic premise being that K^+ tends to move toward the phase where there is the greater fall in pH (Fenn and Cobb, 1934).

Numerous studies have confirmed leakage of K^+ from muscle bathed in saline in vitro (Cook, et al., 1952; Fenn, et al., 1958). However, contrary to Fenn's in vitro study of 1935, it has been shown that plasma potassium concentrations are increased in vivo during respiratory acidosis induced by 30% CO_2 breathing in a variety of species (Youn, Sealy and Harris, 1954; Mackay, 1947; Brown and Goot, 1963; Brown and Mowlem, 1960; Waddell and Butler, 1959). It was postulated that possibly the liver, splanchnic bed or erythrocytes were the source of increased K^+ in plasma during CO_2 induced acidosis (Mackay, 1947). The increment in extracellular K^+ is five to ten times greater than can be explained by hepatic glycogenolysis (Fenn and Asano, 1956). Abdominal evisceration of dogs did not diminish the hyperkalemia produced by 30% CO_2 breathing (Long, Clancy and Brown, 1963). Furthermore, when plasma and whole blood K^+ concentrations were measured in human blood equilibriated in vitro with 20 - 30% CO_2 plasma K^+ rose very slightly (Kilburn, 1965). Thus, the present data suggests that muscle is the major source of elevated plasma K^+ values during respiratory acidosis.

Plasma and whole blood K^+ concentrations increase significantly during muscular exercise both in dogs (Ladé and Brown, 1963) and in humans (Kilburn, 1966). Also, hydrochloric acid infused directly into the bloodstream of dogs causes a significant K^+ concentration rise in the extracellular fluid (Simmons and Avedon, 1959; Schribner and Brunell, 1956; Brown and Goot, 1963). These same investigators have shown that bicarbonate infusion causes a decrease in plasma potassium concentrations.

Adler, Roy and Relman (1965) using rat diaphragm demonstrated

that the acidity of muscle cells is readily influenced by external HCO_3^- as well as CO_2 tension. Their results showed that there was a slight but significant increase in tissue K^+ content in metabolic alkalosis produced by increasing the extracellular HCO_3^- content and respiratory alkalosis produced by decreasing the partial pressure of CO_2. Also there was a slight but significant decrease in tissue K^+ content in metabolic acidosis produced by decreasing the extracellular HCO_3^- content and respiratory acidosis produced by increasing the partial pressure of CO_2.

Fenn, Rogers and Ohr (1968) observed that the Na^+ pump in muscle appears to function better when the Na^+ is being moved into a more acid solution. Contrary to this Adler, Roy and Relman (1965) reported that progressively severe acidosis or alkalosis, either respiratory or metabolic, appeared to cause increasing accumulation of intracellular sodium. Also, Waddell and Bates (1969) employing the findings of a great number of investigators found that there is an increase of Na^+ in the cell relative to the concentration outside the cell when K^+ and H^+ are similarly changing. These observations could be explained by means of a pump model such as that presented in Fig. 2 b.

If alterations in intracellular pH are capable of bringing about changes in K^+ distribution it follows that the converse might be true. Irvine, et al., (1961) found that potassium deficiency caused significant extracellular alkalosis and intracellular acidosis with an increased gradient of H^+ ion across the cell membrane of rat gluteal muscle as measured with the DMO method. Similar observations have been reported

by Sanstone and Muntwyler, (1966, 1967). These results are in direct contradiction to the findings of Miller et al., (1963) who claim that cell pH did not decrease with loss of tissue potassium in the rat diaphragm preparation employing the CO_2 and DMO methods of intracellular pH measurement simultaneously. Hudson and Relman (1962) found that extracellular alkalosis and intracellular acidosis developed in rats on a low K^+ diet could be reversed by injections of KCl and RbCl.

As mentioned previously both muscular exercise and increased pCO_2 are capable of bringing about increased K^+ efflux. Dubuisson (1955) has shown decreased intracellular pH accompanies muscular contraction. Since both acidosis and anoxia are likely to accompany fatigue the effects of fatigue may be simulated by intracellular acidosis and anoxia. A simple means of achieving this practically would be to gass the muscle with a high carbon dioxide, low oxygen gas mixture. Will this result in a similar response in muscle tension to that seen in fatigue and does it also result in a similar change in K^+ flux? Furthermore, if intracellular acidosis is a primary cause of muscle fatigue will factors which affect H^+ ion transport also affect the recovery process?

That a relationship between muscle K^+ and intracellular pH does exist and that this relationship is consistent with the concept of a net $H^+ - K^+$ exchange as proposed by Fenn and Cobb in 1934 seems reasonable. However, whether the net movement of K^+ is directly coupled with H^+ or accomplished by other means is uncertain. If, as predicted by Fenn and Cobb (1934), K^+ does tend to move toward that phase where there is a greater fall in pH, this would only be true if there were some

mechanism which tends to keep the ratio K_i/K_o (inside to outside) equal to H_i/H_o (i.e. a Donnan equilibrium) even though true equality is never really attained. Further support to this idea has been provided by Brown and Good (1963) who demonstrated that the ratio H_i/H_o does change in the same direction in both respiratory and metabolic acidosis thus explaining why potassium concentration rises in the extracellular fluid when an animal breathes high concentrations of CO_2.

The bulk of the evidence presented here suggests that K^+ and H^+ do not obey a Donnan equilibrium and therefore some active transport process exists. This process probably involves some interaction between K^+ and H^+ transport either by a simple direct exchange mechanism or a variation of the $Na^+ - K^+$ pump as described in Fig. 2 b.

b) Changes in the membrane potential

From Table I we see that the normal resting membrane potential of isolated frog sartorious muscle is approximately -94 mv (negative inside). Depolarization of the membrane may be brought about by the application of a negative pulse to a stimulating electrode on the surface of an isolated muscle or by altering the ion balance across the membrane (i.e. mechanical damage, the application of acetylcholine). During depolarization the membrane potential not only decreases to zero but overshoots, that is, it becomes more positive to the inside than to the outside by approximately 30 mv. The events of the action potential may be summarized as follows. At the onset of depolarization membrane permeability to Na^+ increases and Na^+ diffuses into the cell down its electrochemical gradient, this causes a further depolarization of the

membrane which results in a further increase in Na^+ permeability. This chain reaction continues at a rapid rate until the equilibrium potential defined by Nernst's equation ($E = 58 \log Na_o/Na_i$) is reached. After approximately 1 msec. inactivation of the Na^+ current takes place. It is postulated that a Na^+ inactivating mechanism, slower than the chain reaction described above gradually develops restoring the membrane impermeable to Na^+. Recovery of the muscle is further aided by a K^+ current moving down its electrochemical gradient from inside to outside, intensified after the Na^+ current is over. Thus, the K^+ diffusion potential is re-established and the K^+ current returns to normal when the K^+ diffusion potential reaches the value of the K^+ equilibrium potential. The Na^+ - K^+ pump then restores the lost K^+ to the inside and removes Na^+ to the outside of the cell.

In squid axon approximately 3×10^{-12} mole/cm^2 of Na^+ and K^+ cross the cell membrane during a single action potential (Hodgkin, 1958). Hodgkin and Horowicz (1959) studying ionic movements in single muscle fibers observed a net entry of 15.6 ± 1.8 pmole/cm^2 of Na^+ and a net exit of 9.6 ± 0.7 pmole/cm^2 of K^+ per impulse.

The outward flow of current during the falling limb of an action potential is due to a rise in the permeability of the membrane to potassium ions. This increase in K^+ permeability when the potential difference across the membrane is decreased below the resting level is called delayed rectification and is common to both nerve and muscle (Jenerick, 1953, 1959). The membrane of both heart and skeletal muscle also display a rectifying property of opposite direction to delayed

rectification which is called anomalous rectification. Both hyperpolzrizing currents and depolarizing currents below a certain magnitude are capable of evoking anomalous rectification whereby K^+ permeability is decreased by an outward electrochemical gradient and increased by an inward electrochemical gradient. Adrian (1960) concluded that delayed rectification must preceed anomalous rectification. Nakajima et al., (1962) have suggested that a K^+ inactivation process occurs during depolarization and that this process converts delayed rectification into anomalous. Whether this attempt by the muscle to conserve K^+ can be attributed to a special modification of channels through the membrane (Adrian and Freygang, 1962; Hall, Hutler and Noble, 1963) or to differences in permeability of the membranes of the sarcoplasmic reticulum (Adrian and Freygang, 1961, 1962; Hodgkin and Horowicz, 1960) is under further investigation.

c) Changes in the $Na^+ - K^+$ pump

The ability of the cardiac glycoside Ouabain and the cardioactive aglycone strophanthidin to inhibit $Na^+ - K^+$ exchange in skeletal muscle is well documented (Johnson, 1956; Edwards and Harris, 1957; Harris, 1957; Horowicz and Gerber, 1965). Both of these compounds appear to have identical effects on Na^+ and K^+ fluxes when employed in equal concentrations (Sjodin and Beaugé, 1968).

Keynes (1965) found that in freshly dissected frog sartorious muscles treated with 10^{-5} M Ouabain the rate of K^+ flux was decreased less that 0.3 pmole/cm^2/sec. Likewise, Sjodin (1965) found the K^+ flux ratio altered less that 15% upon application of 5 µg/ml of strophanthidin

to freshly dissected frog sartorious muscle. According to the results of Harris (1957) not only is the K^+ uptake in frog muscle reduced 47% by the action of cardiac glycosides but the potassium efflux is increased approximately 30%.

An explanation for the variation in the above observations has been provided by Sjodin and Beaugé (1968). They found that "low sodium" muscles (5 mmoles/kg fiber of intracellular Na^+) treated with 10^{-5} m strophanthidin reduced K^+ influx by about 5% with no change in K^+ efflux. However, "high sodium" muscles (15 - 30 mmoles/kg fiber of intracellular Na^+) exhibited not only a 45% reduction in K^+ influx but an increased K^+ efflux of 70 % when treated with 10^{-5} m strophanthidin. Since freshly dissected muscles are gaining sodium during an experiment and since the Ouabain and strophanthidin sensitivity of Na^+ and K^+ fluxes changes with increasing internal sodium concentration, these findings of Sjodin and Beaugé may explain why a variety of divergent results were obtained by previous investigators. Exactly how 10^{-5} m strophanthidin brings about a rather large increase in K^+ efflux when the internal Na^+ concentration becomes high is unknown. The blocking of the $Na^+ - K^+$ pump also has an indirect effect on intracellular pH (Bondani and Withrow, 1965) as one would expect from our previous discussion.

4. Factors which effect ion flux - the bulk-phase theory

According to the bulk-phase theory the resting potential measured by means of intracellular microelectrodes is due to damage of the protoplasm and decomposition of the protein-electrolyte complex. Likewise, the action potential observed during excitation of the nerve or muscle

fiber is accompanied by a definite kind of change in the labile protein-water-ion matrix which results in this system losing its adsorptional affinity for K^+ ions and acquiring an affinity for Na^+ ions. The excess of the action potential over the resting potential is explicable on the basis that more K^+ ions are released from the bound state in the excited portion of the protoplasm than occurs upon mechanical damage, i.e. upon the introduction into the fiber of a glass electrode.

Furthermore, since a multiplicity of choices of counterions for fixed anions and cations exists (Ling, 1962) the possibility of exchange between H^+ and K^+ can be accounted for on the basis of the bulk-phase theory. Once the protoplasm passes out of the excited state or the damage is liquidated, the previous state of the complex is restored at the expense of metabolic energy. Once restored the protein-ion matrix loses its affinity for Na^+ which is exchanged for K^+. Any breakdown in metabolism (i.e. anoxia, metabolic inhibitors, etc.) hinders the redistribution of inorganic substances between the cell and the medium (Troshin, 1966).

5. Kinetics and methods of ion flux measurement

Just as there are two schools of thought regarding the nature of intracellular K^+ retention, there are also two theories concerning ion movements from the interior to the exterior of the cell. The models which embody the basic features of these contrasting theories may be called the diffusion model and the permeability model (after Sjodin and Henderson, 1964).

The diffusion model proposes that whole muscle is an assemblage of cellular units in which simple permeability kinetics are not obeyed due to the presence of large modifications caused by slow diffusion intracellularly. Troshin (1960, 1966) has found three exponential components in K^+ and Na^+ turnover curves in muscle; namely, a fast fraction representing the extracellular space, a medium fraction consisting of ionized intracellular K^+ and a slow fraction of bound K^+. Likewise, Simon (1960) has found that the results of tracer studies can be adapted to a three phase system; one phase being the extra-cellular' space of the muscle, the second being an intracellular free phase and the third an intracellular bound phase.

Those which uphold the permeability model regard whole muscle as a population of single cellular units each obeying simple first-order permeability kinetics with minor modifications. Hodgkin and Horowicz (1959) found the ^{42}K efflux from single muscle fibers follows such a single exponential law. It has been argued that due to the variability of fiber sizes, a whole muscle consisting of a varied population of fibers cannot be expected to follow these same first-order permeability characteristics. However, Sjodin and Henderson (1964) studying K^+ efflux from frog sartorious muscle employing both ^{42}K tracer and direct K^+ analysis found that whole muscle followed closely the first-order permeability process obeyed by single constituent fibers. Therefore, if fiber variability can be ruled out as a source of flux deviation from a single exponential law the only remaining source of deviation would have to be some form of intracellular K^+ binding. for this reason, the study

of ion flux is of special interest to both the membrane and bulk-phase theoriests. The net K^+ efflux measured by a number of authors employing the following methods is illustrated in Table III.

1) Terminal method (Fenn and Cobb, 1934)

This method consists of removing paired muscles, determining the K^+ content of one immediately, soaking the other in an experimental solution (high K^+, low K^+, high pH, low pH, etc). and after a given period of time determining the K^+ content of this muscle. By means of comparing the resulting K^+ content of the experimental muscle with that of the control the net gain or loss of K^+ is determined. The drawbacks to this method are that one must assume equality of K^+ concentration in the paired muscles, only one experiment can be performed on a single muscle, only one value is obtained over a fairly long time period, and kinetic studies are not possible.

2) ^{42}K tracer technique (Harris, 1957; Hodgkin & Horowicz, 1959,etc.)

In this method muscles are allowed to equilibriate in ^{42}K Ringer's solution for a given period of time. K^+ influx may be measured by the uptake of ^{42}K by the muscle and efflux measured by the rate of ^{42}K loss by the muscle into a non radioactive solution. Both influx and efflux may be carried out under a variety of experimental conditions. However, if done properly two tracer isotopes are required, one to trace influx and one to trace efflux. Also, there is disagreement as to whether ^{42}K is totally exchangeable with the intracellular K^+.

3) Direct method

This method consists of immersing the muscle in a bath and

TABLE III

Net K^+ Efflux values for resting and active muscle

Author	K^+ external (meq/l)	Net efflux (pmole/cm^2/sec)	Method	Preparation
i) Resting State				
Hodgkin & Horowicz (1959)	2.5	1.0 - 3.4	^{42}K - tracer	single fiber, frog semitendinosus
Sjodin & Henderson (1964)	5.0	0.299	^{42}K - tracer	whole muscle, frog sartorious
	2.5	0.633	direct K^+meas't.	
Sjodin (1965)	0.21	3.27 - 5.33	^{42}K - tracer & direct K^+meas't.	whole muscle, frog sartorious
Harris (1957)	2.0	1.95 (1st 100 min) .65 (next ")	^{42}K - tracer	whole muscle, frog sartorious
Keynes (1954)	2.5	0.70	^{42}K - tracer	whole muscle, frog M. extensor longus, Dig.1v
	5.0	0.30		
Lucier (thesis)	2.5	0.78	direct K^+ measurement	whole muscle, frog sartorious
ii) Active State		(pmole/cm^2/impulse)		
Creese, Hashish & Scholes (1958)	5.9	10	^{42}K - tracer	isolated rat diaphragm
Hodgkin & Horowicz (1959)	2.5	9.6	^{42}K - tracer	single fiber, frog semitendinosus
Lucier (thesis)	2.5	8.6	direct K^+ measurement	whole muscle, frog sartorious

measuring the K^+ efflux into the surrounding solution and the K^+ remaining in the muscle usually by means of flame photometry (thus, K^+ lost by muscle = K^+ gained by surrounding medium). Unfortunately, changes are difficult to detect due to the relatively large volume of bath compared with the small amount of efflux. The direct method could however, give clear and unambiguous results if the technical difficulties could be overcome, providing a new approach to the study of ions in muscle.

An analysis of the steady state flux in the presence of different external electrolyte concentrations and ion pump inhibitors should provide evidence to test current equivalent circuits of the muscle membrane in the resting state. The direct measurement of transient changes in ion flux during muscle activity could provide data to test the theoretical basis of the change from rest to activity in muscle. It therefore, seemed important to look into the difficulties of measuring ion flux directly, to see if a suitable method could be developed to apply to these problems.

6. Statement of the problem

The problem herein considered may be stated as follows:

i) To develop a direct method of measuring continuous ion flux and apply this method to the measurement of net K^+ efflux in frog sartorious muscle.

ii) To relate the results of this method of measurement to those made by other means.

iii) To investigate changes in K^+ flux which accompany muscular

activity.

iv) To relate the observed K^+ flux changes to the theoretical basis of ion flux.

CHAPTER II

Methods

A modification of the direct method was employed in this study to measure net K^+ efflux. The main difficulty in the direct measurement of potassium efflux is the small concentration differences that have to be detected. Clearly the smaller the volume of the external bathing solution the greater will be the concentration change due to a given K^+ efflux. If the external volume could be reduced to the extracellular fluid between the fibers and a thin surface film then the effects of a given efflux could be amplified considerably. While this seems the answer to the main problem it leads to three other problems which must be faced:

1) The extracellular fluid concentration changes due to the efflux may themselves alter the electrochemical gradient between the inside and outside of the cells.

2) A continuous sampling system providing at least 100 microlitre samples from the surface film is required.

3) Small losses of water from the surface film by evaporation could lead to apparent net fluxes.

An appropriate surface film can be formed by superfusing a muscle suspended in a gas phase from a constant flow source. If the rate of flow is appropriately adjusted then the first two problems can be overcome since the surface film is constantly exchanged. To solve the third

problem the water vapour pressure of the gas phase has to be carefully controlled. Net gain or loss of water can be detected by a substance in the perfusing fluid which will not penetrate the membrane barrier. ^{14}C Inulin was used for this purpose. At the same time this permitted measurement of the surface film and extracellular fluid volume in the muscle at the end of each experiment.

1. Preparation of muscle

Unblemished sartorious muscles of Rana pipiens in good nutritional state were used in all experiments. The frogs were kept in the cold for several weeks prior to use, thus minimizing seasonal variations. Before dissection each animal was injected in the ventral lymph sac with 3 mg. of D-tubocurarine and left in the cold for a further thirty minutes. This procedure seems to reduce the variation between paired sartorious muscles as described by Marechal and Mommaerts (1963). The pelvic bone was split with a thin stainless steel razor blade, each muscle carefully dissected and placed in oxygenated Ringer's solution. A small hole was drilled in the chip of pelvic bone with a No.22 hypodermic needle. The muscle was then placed on a Teflon frame (Fig. 3 b) so that the tendinous end of the muscle was attached to the base of the frame by means of a loop of surgical suture.

2. Preparation of solutions

All solutions used in the experiments were isotonic. They were freshly prepared using deionized distilled water and Fisher Certified Reagents. To avoid precipitation of Ca^{++}, $CaCl_2$ was added very slowly

TABLE IV

Composition of solutions (mM)

Solution	K^+	Na^+	Ca^{++}	Cl^-	HCO_3^-	Ouabain	Sucrose
A	2.5	122.6	1.8	120.1	3.6	-	-
B	2.5	-	1.0	-	4.5	-	242
C	2.5	20.0	1.8	20.0	4.5	-	202
D	2.5	50.0	1.0	50.0	4.5	-	142
E	2.5	121.0	1.8	102.1	25.0	-	-
F	2.5	121.0	1.8	126.9	0.2	-	-
G	2.5	121.0	1.8	102.1	25.0	$2x10^{-2}$	-
H	2.5	121.0	1.8	102.1	25.0	10^{-1}	-
I	2.5	122.6	1.8	120.1	3.6	10^{-2}	-
J	-	-	-	-	-	-	242

Fig. 3 a

Division of muscle for measurement of surface area, thickness and mean cross-sectional area.

Where W_1 and L = two sides

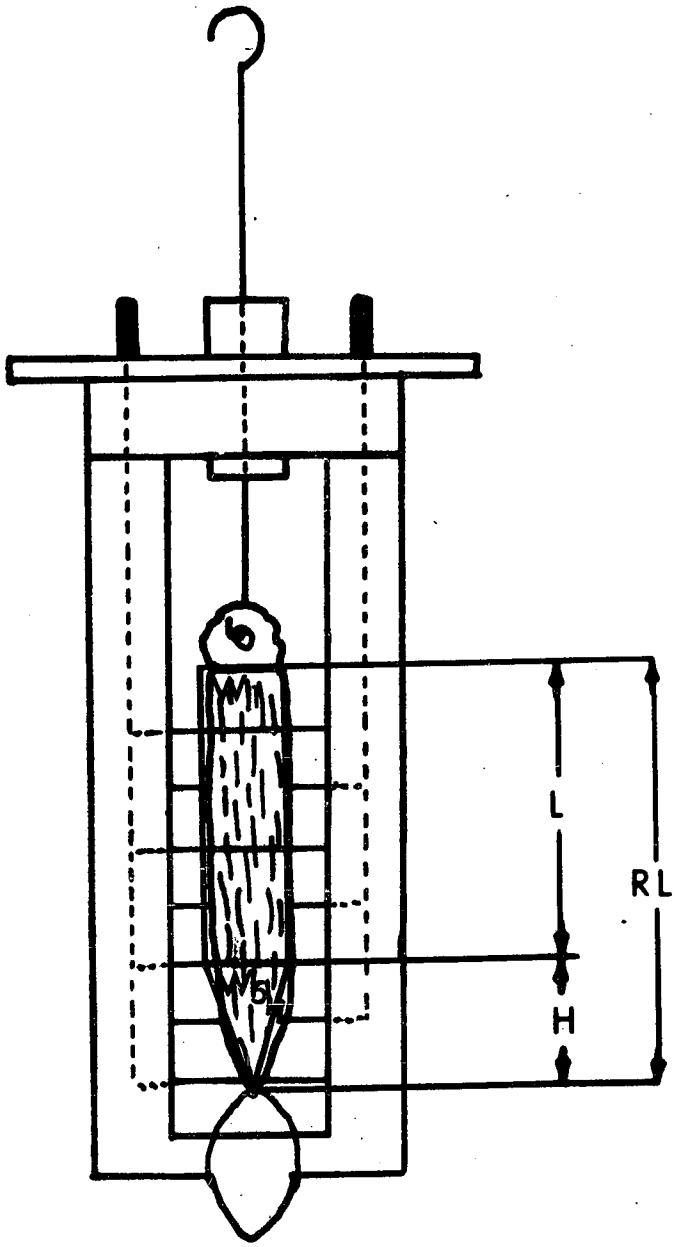
W_5 = base of triangle

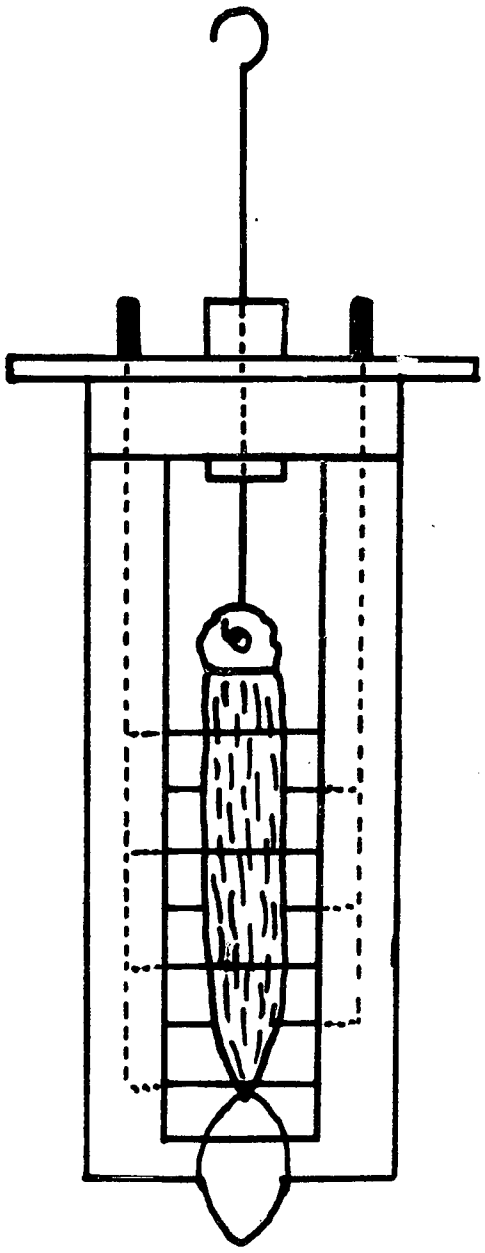
H = height of triangle

RL = rest length of muscle

Fig. 3 b

Muscle mounted on Teflon frame


L
RL
H



and the solutions were shaken continuously. All solutions employed were bicarbonate buffered with the appropriate percentage of CO_2 in O_2 used to obtain the desired pH levels. The pH values of the solutions were measured with a Radiometer pH meter, either right after gassing or at the end of experiments. During experiments in which pure gases or two mixtures were mixed to obtain a desired pH value the pH was continuously monitored throughout the experiment. During this time the pH variation was less than ± 0.2 unit. The composition of the solutions is shown in Table IV. Enough ^{14}C-Carboxyl-Inulin was added to each solution (except sucrose solution J) to give at least ten thousand counts per minute in one hundred microlitres of the solution.

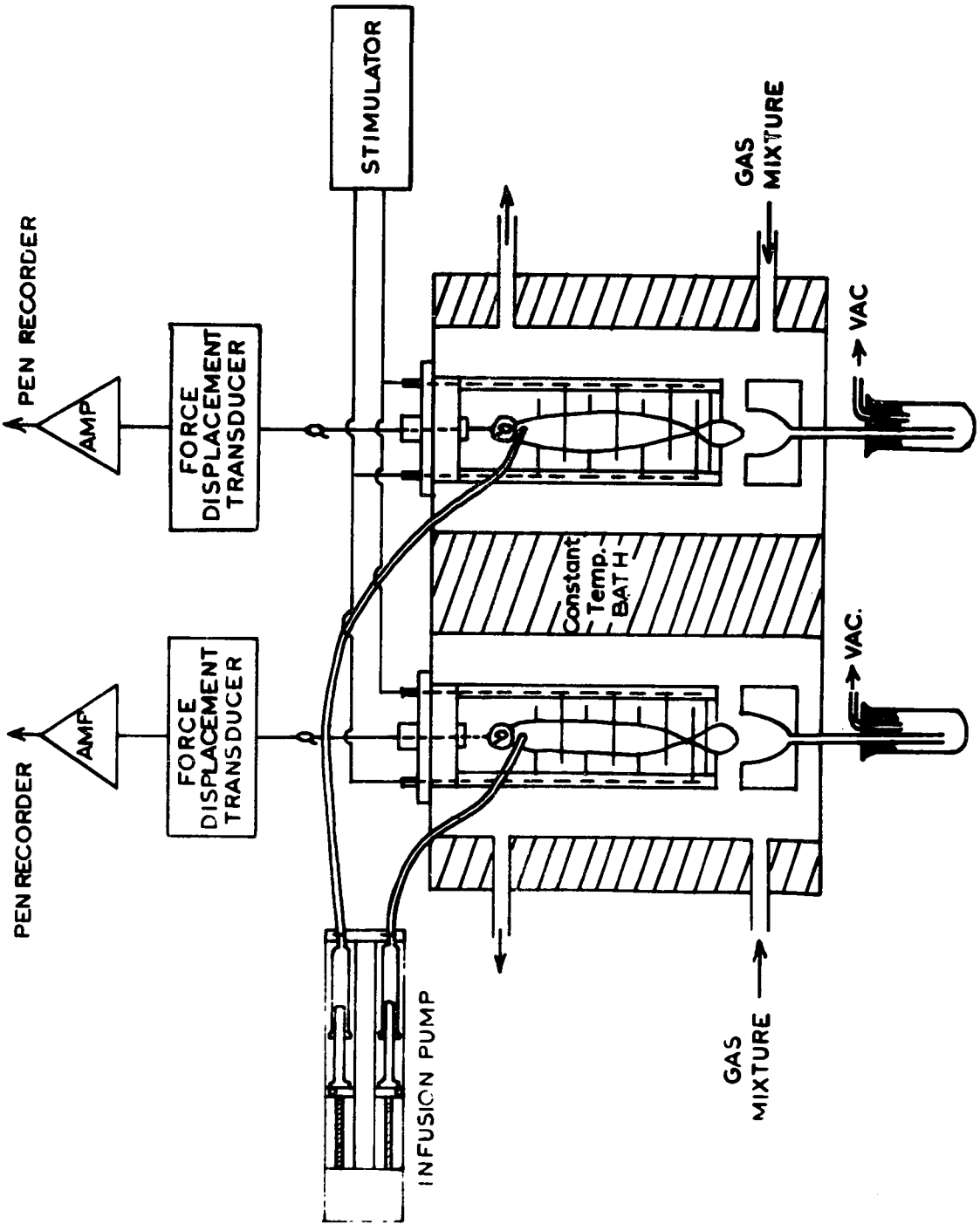
3. Description of apparatus

The apparatus was essentially as shown in Fig. 4.

Tension was recorded on a Grass Model 7 Polygraph employing Grass Force-Displacement Transducers Model FT .03C. This transducer used four bonded strain gauges to form a bridge connection to measure the strain produced on a cantilever beam when a force was applied to it by the muscle. The maximum working range of this instrument was 0.01 to 200 grams when used with a spring. Calibration was accomplished by hanging 10, 20 and 40 gram weights on the lug for preparation attachment. The balance voltages on the preamplifier required for making any one of several 10 gram steps of tension increase the new baseline zero value were checked and recorded before each experiment. Using any one of these values during the experiment permitted the expansion of the

Fig. 4

Schematic representation of apparatus assembly.


PEN RECORDER
AMP
FORCE DISPLACEMENT TRANSDUCER
STIMULATOR
GAS MIXTURE
VAC
Constant Temp. BATH
VAC.
PEN RECORDER
AMP
FORCE DISPLACEMENT TRANSDUCER
INFUSION PUMP
GAS MIXTURE

tension recording in a given range with a resultant measurement of greater accuracy.

The stimulator, a Grass Model S4, was connected to the platinum electrodes in the muscle frames as shown in Fig. 4. The voltage used in all cases was that required to give maximum twitch tension response.

Superfusion of the muscles was accomplished by means of a Harvard Infusion/Withdrawal Pump, Model 940. Fitted with two 5 cc hypodermic syringes, this pump was capable of delivering the superfusion solution within a range of 0.0136 to 6.8 ml/min. Both syringes were fitted with No. 22 blunt hypodermic needles. One piece of teflon spaghetti tubing (I.D. .027 in) approximately 45 cm. in length conducted the solution from the syringe to a liquid interface on the muscle. The tubing passed through a small hole in the face plate of the muscle chamber and was held in place by means of a small piece of plasticine.

Before entering the muscle chamber all gases used were saturated with normal Ringer's solution during passage through Pyrex Gas Washing Bottles immersed in a Grant thermostatically controlled cooling bath maintained at 20^{0}C. The muscle chambers were also maintained at this temperature by means of a pump which circulated water between the bath and three sides of the muscle chambers. The flow of gas to the Washing Bottles was regulated by a needle valve in series with a rotometer type gas flow meter (Roger Gilmont Instruments, Inc. distributed by Cole-Parmer).

The superfusion solution was collected in a teflon cup inside the chamber at the base of the muscle holder. The contents of this cup

was periodically withdrawn under negative pressure into small Blood Storage Tubes (Cutler, 25 x 10 mm). After a given collection period these tubes were interchanged; the full ones containing the effluent were covered with a thin sheet of Parafilm until pipetting.

4. Potassium and ^{14}C Inulin determinations

One hundred microliters of each effluent sample were diluted to 5 ml with distilled and demineralized water in preparation of K^+ determination in the flame photometer. When Na^+ was also determined fifty microliters of effluent were diluted to 10 ml. The pipettes used were lambda, Lang-Levy type supplied by Canlab and calibrated to a single volume within tolerances of 0.5 to 1. All volumetric glassware used to measure or dilute the solutions for analysis was Kimax Class A, Glassware of borsilicate glass which has a low alkali content and no calcium present. To eliminate potassium and sodium contamination of the glassware an extensive washing procedure was adopted which included at least a 24 hour soak in .1 N HCl and thorough flushing with distilled and deionized water.

All K^+ and Na^+ determinations were carried out on a Jarrell-Ash Flame Photometer connected to a Sargent Model 5R recorder. K^+ determinations were carried out at a wave-length setting of 7665.5 A^o and Na^+ determinations at 5891.0 A^o. Before each determination, calibration curves for Na^+ and K^+ were drawn from the appropriate standards. These were chlorides of the grade previously described in concentrations ranging from 0.01 - 0.10 mM for K^+ and 0.02 - 0.12 mM for Na^+. These standards were made up in distilled and deionized water and stored in

tightly sealed polyethylene bottles

To determine if the accompanying ions in the effluent would have any effect on the photometric determination of K^+ a set of standards was made up of K^+ free Ringer's solution in place of distilled and deionized water. The K^+ concentration of these solutions was then determined in the usual manner. From the results illustrated in Fig. 5 it is evident that the accompanying ions in the Ringer's solution have no significant effect on the K^+ determination.

One hundred microliters of each effluent sample was placed in 10 ml. of Bray's solution in preparation for counting in a Nuclear Chicago Mark I Liquid Scintillation System. One liter of the Bray's Solution employed consisted of the following constituents:

60	gm	Napthalene
4	gm	PPO (2,5-diphenyloxazole)
0.2	gm	Dimethyl POPOP {1,4 bis-2-(4 methyl-5-Phenyloxazolyl)-Benzene}
100	ml	Methanol
20	ml	Ethylene Glycol
		1,4 Dioxane to 1 litre.

The PPO and Dimethyl POPOP were supplied by Picker Nuclear of Ottawa. All other reagents were "Baker Analyzed" Reagents. The 1,4 Dioxane contained a peroxide inhibitor.

5. Experimental procedure

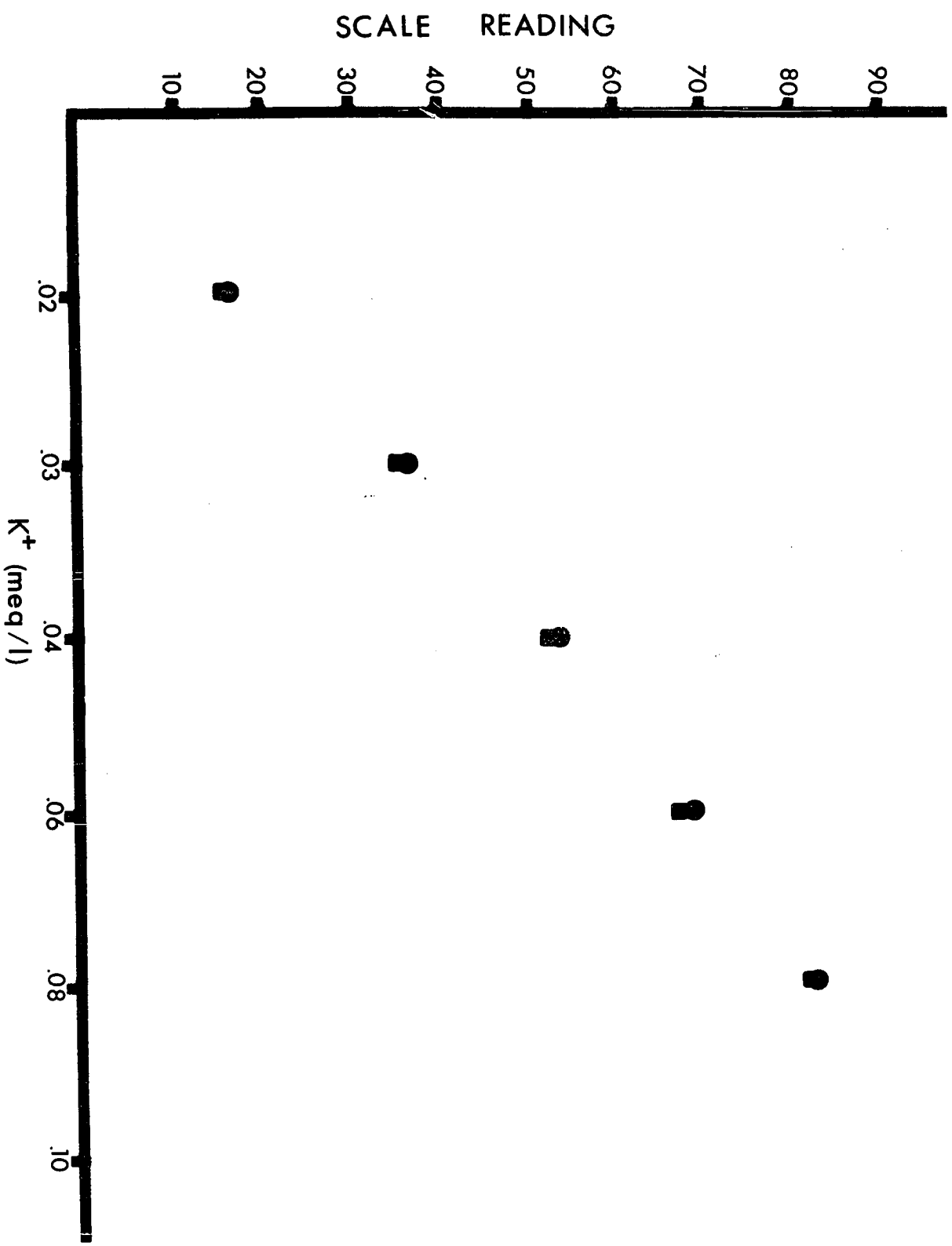
After dissection and mounting, the muscles were placed in an oxygenated bath of the superfusion fluid maintained at a temperature of

Fig. 5

Flame photometer calibration curve.

Where: ● represent varying K^+ concentrations in K^+-free Ringer

■ represent varying K^+ concentrations in distilled and deionized water

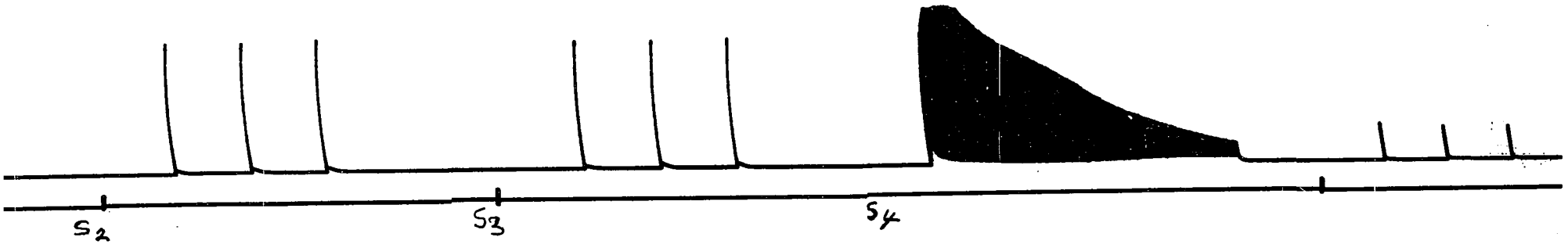

SCALE READING
10
20
30
40
50
60
70
80
90
.02
.03
.04
.06
.08
.10
K+ (meq/l)

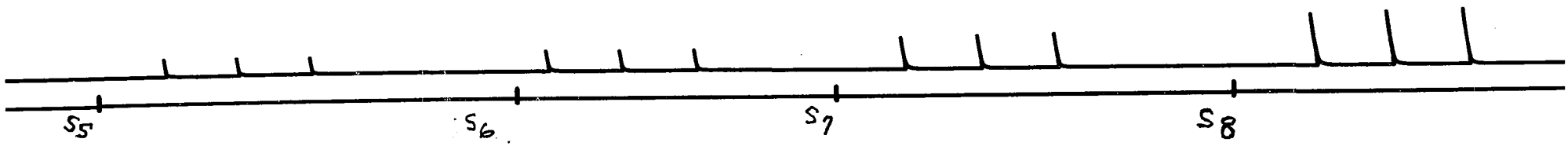
20°C for a period of 30 minutes prior to their introduction into the muscle chambers. Once positioned inside the chambers, the muscles were allowed to equilibriate a further 30 minutes with the superfusion fluid flowing over them at a rate of 0.0136 ml/min. The effluent collected during this time was discarded. The first collecting tube was then set in place and the muscle given three test shocks approximately forty-eight seconds apart. At the end of twenty minutes the collecting tubes were changed and the three test shocks repeated. This was continued until the tension developed in two successive sets of test shocks were equal. At that time the muscles were stimulated to fatigue. This was accomplished by replacing the three test shocks with two shocks per second for 200 seconds at the beginning of one twenty minute collection period. For the duration of the experiment the sets of test shocks were resumed (see Fig. 6).

At the end of the experiment the muscles were washed with isotonic sucrose solution (Solution J, Table VI) for two minutes at a superfusion rate of 0.68 ml/min. The volume of this effluent was determined and 100 microliters of it placed in 10 ml of Bray's solution for ^{14}C Inulin counting. This provided an estimate of the surface fluid on the muscle. The muscles were then removed from the chambers, cut free of the chip of pelvic bone and loop of surgical suture, blotted on Whatman No. 2 filter paper, quickly weighed on a Mettler balance and homogenized in a Bronwill Biosonik homogenizer. The homogenate was then made up to 5 ml. with distilled and deionized water. One hundred microliters of this was further diluted to 5 ml. for K^+ determination

Fig. 6

Sample polygraph recording showing test shocks and stimulation to fatigue. Intervals between sets of test shocks - 20 minutes with 48 second interval between each test shock; stimulation to fatigue - 2 shocks per second for 200 seconds.


S2
S3
S4


S5
S6
S7
S8

and one hundred microliters placed directly in 10 ml. of Bray's solution for ^{14}C Inulin counting as described earlier. This provided a value for the extracellular fluid volume of the muscle.

Measurements -

The rest length of all muscles was measured in situ before dissection and stretched to this rest legnth when placed on the muscle holder. In order to facilitate measurements the muscle was divided into a rectangle with an adjoining triangle (see Fig. 3 a). All measurements were made in millimeters. The formulae used for the calculation of muscle surface area, thickness and mean cross sectional area are as follows:

$$S.A. = \frac{W_1 + W_5}{2} \times L + \tfrac{1}{2}H \times W_5$$

$$T. = \frac{\text{Wt. of muscle (mg)}}{\text{S.A. (mm)}^2}$$

$$M.C.A. = \frac{W_5 + W_1}{2} \times T$$

Where:

W_1 & L = two sides of the rectangle

W_5 = base of the triangle

H = height of the triangle

CHAPTER III

Results

1. Critical survey of experimental methods

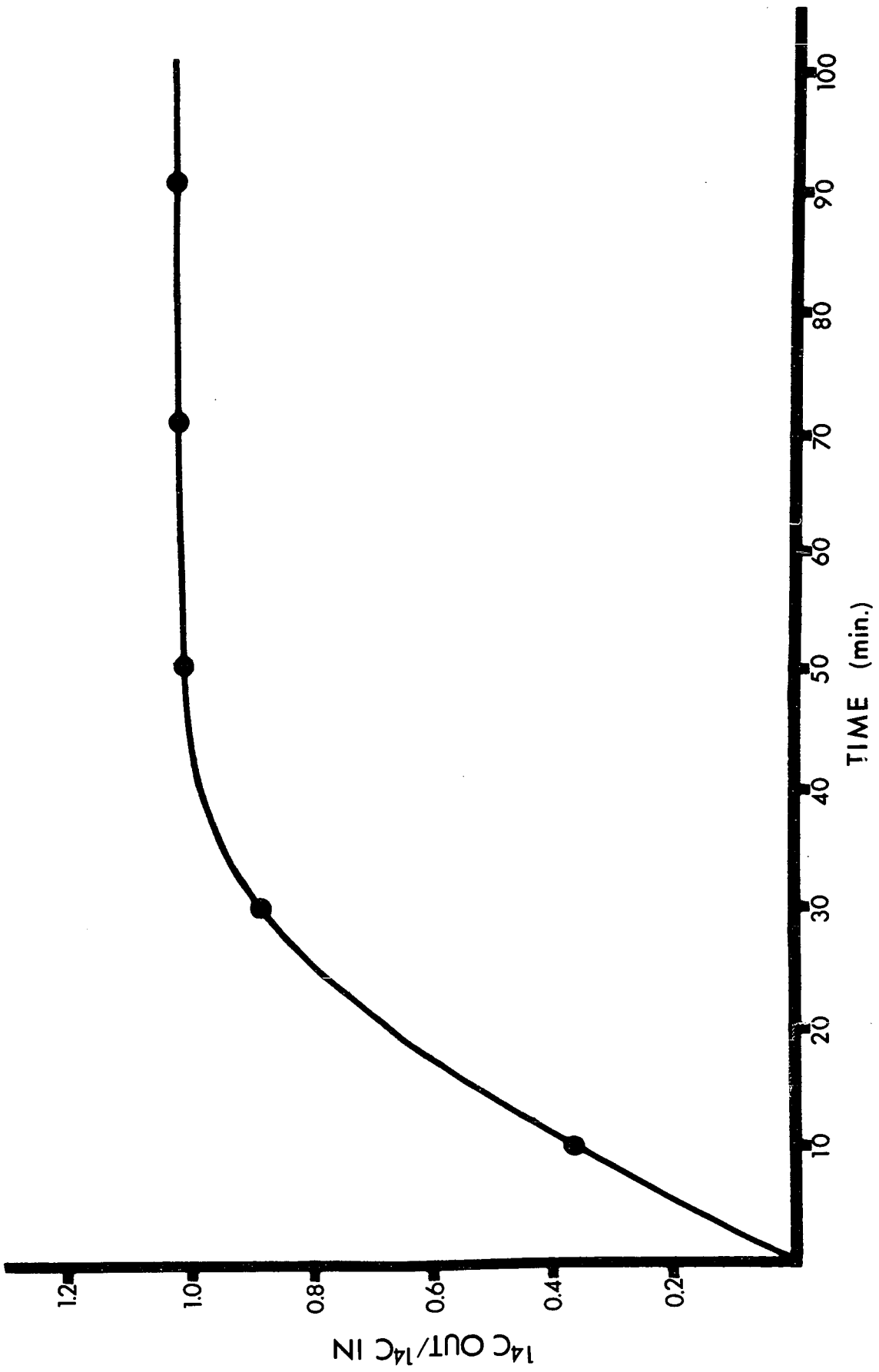
Since the development of a new technique to measure continuous ion flux was an integral part of this project the first section of the results will deal with some of the basic problems encountered during the development of this thin layer superfusion method.

a) Flow rate

The rate at which the superfusion fluid was to be passed over the muscles and the duration of the collection intervals was of upmost importance in this technique. The volume of the collected effluent for each time interval had to be large enough to provide at least two one-hundred microliters portions (one for K^+ determinations and one for ^{14}C Inulin counting). The sample size could be altered by increasing or decreasing the duration of collecting period or by increasing or decreasing the superfusion flow rate. However, if the time interval between collection periods was too great, short duration changes in K^+ efflux rate would go undetected; similarly if the flow rate was too rapid the volume would be too large to detect the slight changes in K^+ efflux anticipated. After a large number of trial experiments varying both collection interval and flow rate, it was found that a collection period of twenty minutes at a flow rate of 0.0136 ml/min proved optimal. The sample size collected under these circumstances was just slightly over

Fig. 7

Rate of ^{14}C Inulin uptake by extracellular fluid.


1.2
1.0
0.8
0.6
0.4
0.2
14C OUT/14C IN
10
20
30
40
50
60
70
80
90
100
TIME (min.)

two hundred microliters. This flow rate also allowed a complete experiment to be carried out (average duration 210 minutes) without having to refill the 5 ml. delivery syringes, thus eliminating a possible source of error.

A number of studies employing ^{14}C Inulin as a tracer revealed that there was no significant difference in the amount of superfusion fluid delivered per unit time by the two 5 ml. delivery syringes driven at a constant rate.

b) Water balance

Any gain or loss of water by the superfusion fluid would alter the concentration of K^+ determined in the small volume of effluent collected and result in fluid and electrolyte shifts between the muscle and the superfusion fluid. In order to reduce this source of error all solutions were made isotonic and all gases were saturated with normal Ringer's before passage into the muscle chambers as mentioned previously. As an additional safeguard sufficient ^{14}C- Carboxyl-Inulin was added to each superfusion solution to give at least ten thousand counts per minute in one hundred microliters of the collected effluent. By monitoring the ratio of ^{14}C in the effluent/ ^{14}C in the superfusion fluid any volume changes could be easily detected. Since Inulin passes into the extracellular fluid of the muscle it must first equilibriate with this space before the previously mentioned ratio will become unity. Fig. 7 represents the uptake of ^{14}C Inulin by sartorious under the experimental conditions described here. It can be seen that at the end of 30 minutes, over 80% of the extracellular space is exchanged with

complete exchange after 50 minutes. This is in keeping with the observations of Ling and Kromash (1967) who found the ^{14}C Inulin required 19 minutes to reach 90% exchange in the extracellular space of thin muscles and 41 minutes to reach 99% exchange. Since the muscles were superfused for 30 minutes prior to the start of each experiment by the time the first sample of effluent was collected the muscles had been in contact with the ^{14}C Inulin for 50 minutes and ratio ^{14}C out/ ^{14}C in was almost unity.

c) Determination of compartment size

The volume of surface fluid covering the muscle was determined from the ^{14}C Inulin concentration in the isotonic sucrose solution used to rapidly wash the muscle at the end of the experiment. The average volume of the surface water was 61.7 microliters (see Table V).

The concentration of ^{14}C Inulin in the muscle homogenate provided an estimate of the extracellular fluid volume. Any error introduced by the uptake of ^{14}C Inulin by the muscle fibers would be negligible in this case due to the short duration ($\leq$ 4 hours) of each experiment (Tasker, et al., 1960). Table VI illustrates the volume of extracellular space expressed as a per cent of muscle weight as determined by a number of authors.

Subtracting the weight of the extracellular fluid volume from the wet weight of the muscle provided a value for the mass of the muscle fibers. Employing this value and taking the density of frog sartorious muscle as 1.05 gm/cm^2 (Hill, 1965), the intrafiber volume could be calculated (see Table V).

TABLE V

Volumes of the various muscle compartments

	Volume per muscle	No. of observations	S.D.	S.E.
Collected fluid	.272 ml (per 20 min. collection period)			
Surface fluid	.0617 ml	40	.033	.0037
Fiber fluid	.1028 ml	40	.042	.0066
	(.830 ml/gm)	40	.030	.0049
Extracellular fluid	.0147 ml	40	.0041	.0007
	(.127 ml/gm)	40	.033	.0052

TABLE VI

Volume of inulin space as found by various authors

Author	No. of observations	Inulin space % (vol/wt)
Tasker, et al. (1959)	142	24.8 - 0.83 (S.E.)
	20	16.9 - 0.82 (S.E.)
Edwards and Harris (1957)	10	16 - 26
Desmedt (1953)	7	11.2 - 13.9
Ling (1967)	10	10.3
Bozler (1967)	24	17 - 2.4 (S.D.)
	16	23 - 3.4 (S.D.)
	12	18 - 3.1 (S.D.)
	10	16 - 2.6 (S.D.)
Harris (1957)	22	19
Johnson (1956)	56	18 - 0.5 (S.E.)
Lucier (thesis)	14	12.3 - 3.9 (S.D.)
	12	12.2 - 3.3
	14	13.2 - 2.6

d) Diffusion study

The rate of net K^+ flux from a muscle fiber into the extracellular fluid can only be determined if the relationship of the actual flux between the cells and the extracellular fluid and the apparent flux measured in the whole system is known. There are three possible rate limiting steps in the movement of K^+ from the extracellular fluid to the collected effluent. These are: diffusion within the extracellular space, diffusion from the extracellular space to the surface water through the connective tissue layer and the superfusion flow rate. Since the superfusion flow rate could be adjusted to fit the needs of the sampling procedure this problem was investigated as follows.

Net efflux from the preparation was measured in the non-steady state when efflux rates from the whole system could be measured at a series of decreasing mean extracellular fluid concentrations. Sodium was chosen as a suitable cation because of its relatively low membrane permeability and the fact that the low intracellular Na^+ concentration results in a small flux rate between the intra- and the extracellular compartments. Thus, if the initial extracellular sodium level is known and if the muscle is washed in an isotonic solution containing no sodium the net flux can be measured and the mean Na^+ concentration remaining can be calculated at any instant. If the flux rates and corresponding concentration differences between the surface fluid and mean extracellular fluid are known an arbitrary diffusion index (D) can be calculated. This can be measured in units of flux rate/concentration difference and is ameasure of the "diffusing efficiency" of

the system. While it ignores concentration gradients within the muscle it seems to offer a useful practical measurement for determining the magnitude of the error arising from the concentration changes in the extracellular fluid arising as a result of a net flux from the whole system.

Preliminary experiments indicated an additional advantage in using Na^+ to estimate the diffusion index. It was found that twitch tension decreased in a regular, though near linear fashion with extracellular Na^+ concentration so that with a calibration curve an independent check of mean extracellular Na^+ concentration could be made.

Paired muscles were placed in the apparatus and both were superfused with normal Ringer's solution (Solution A, Table IV) at a rate of 0.0136 ml/min. Both muscles received a test shock every 60 seconds for the duration of the experiment. Samples of the effluent were collected at 20 minute intervals as usual. At the end of 120 minutes, muscle A was rapidly washed for 2 minutes with isotonic sucrose (SOlution B, Table IV), blotted, weighed, and homogenized as previously described. At the end of 150 minutes muscle B was superfused with isotonic sucrose solution at a flow rate of 0.194 ml/min. Effluent samples were collected every 2 minutes for 30 min. At the end of this time the flow rate was decreased to 0.0194 ml/min and samples collected every 20 minutes for 60 minutes. The superfusion solution was then changed to 20 meq/1 NaCl Ringer (Solution C, Table IV). At the end of 30 minutes the superfusion solution was changed again to 50 meq/1 NaCl Ringer

(Solution D, Table IV) and at the end of another 30 minutes the superfusion solution was changed to normal Ringer (Solution A, Table IV). At the end of 30 minutes the muscle was removed from the apparatus, blotted, weighed, and homogenized as previously described. All calculations for this experiment are based on the model described in the Appendix. The extracellular volume of muscle B as calculated from the ^{14}C Inulin count of the homogenate of muscle A was approximately 27 microliters. Since the extracellular Na^+ concentration is 120 meq/l the total extracellular Na^+ of muscle B would be 3,240 Neq. Fig. 8 describes the washout curve. It is interesting to note that the curve starts to level off at approximately the Na^+ level determined for the extracellular space (vertical line). This would indicate that at this point the Na^+ content of the extracellular space was exhausted and that Na^+ started to diffuse out of the muscle fibers at a much slower rate.

The diffusion index for the washout of Na^+ from the extracellular space was found to be 8 neq/min per gradient of /meq/liter. This value is of sufficient magnitude to show that diffusion through the extracellular space and the connective tissue layer to the superfusion solution is not a serious rate limiting step in this technique of measuring continuous ion flux.

2. Effects of stimulation to fatigue

In order to determine the effect of stimulation to fatigue on K^+ efflux a series of six experiments were performed on paired muscles employing normal Ringer's solution (Solution A, Table IV) gassed with

Fig. 8

Plot of F x Ca versus Y.

Diffusion Study.

See text for explanation.

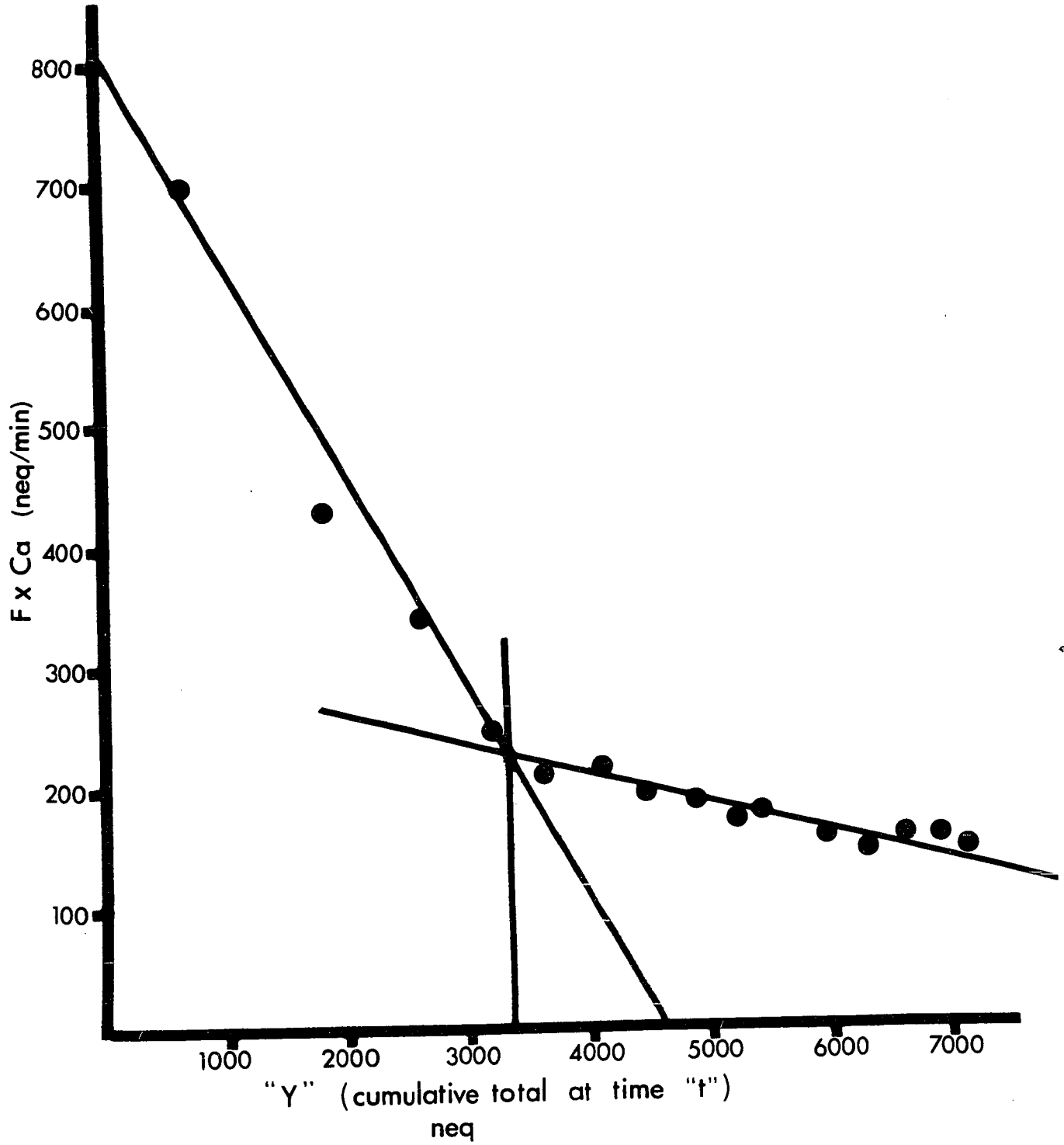

800
700
600
500
400
300
200
100
F x Ca (neq/min)
1000
2000
3000
4000
5000
6000
7000
"Y" (cumulative total at time "t")
neq

Fig. 9

Results of one of a series of six experiments performed to study effects of stimulation to fatigue on muscles superfused with normal Ringer's solution (Solution A, Table IV) gassed with 1% CO_2.

Where ● represent muscle A

■ represent muscle B

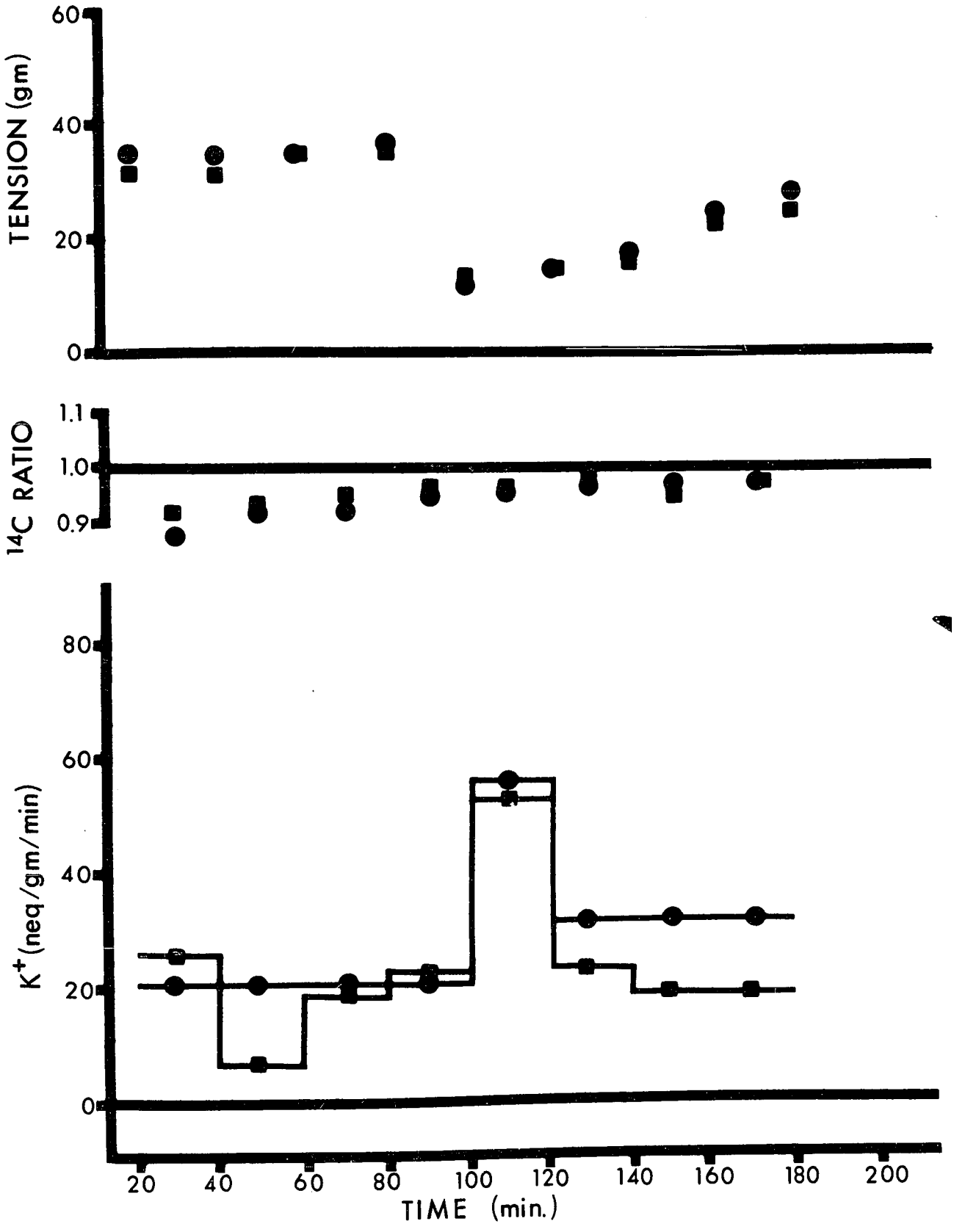

TENSION (gm)
60
40
20
0
^{14}C RATIO
1.1
1.0
0.9
K^+ (neq/gm/min)
80
60
40
20
0
20
40
60
80
100
120
140
160
180
200
TIME (min.)

TABLE VII

Effect of constant stimulation on K^+ efflux

State	No. of Muscles	No. of observ'n	Mean K^+ loss Neq/gm/min	S.D.	S.E.	P.
Resting	12	36	24.83	16.93	2.82	.001
Activity	12	12	57.79	31.14	8.98	.001
Recovery	12	12	34.09	14.89	4.29	

Effect of constant stimulation on twitch tension

	No. of Muscles	No. of observ'n	Tension (g)	S.D.	S.E.	P	%
Before	12	36	35.1	9.68	1.61	.001	100
End of stimul'n	12	12	8.8	3.46	1.00	.001	25.1
Recovery (40 min)	12	12	16.8	4.82	1.33		47.9

1% CO_2 to give a pH of 7.14. (See Fig. 9 for a typical experiment representation in this series.) Upon constant stimulation the twitch tension decreased from 35 grams to approximately 10 grams following stimulation for 200 seconds at two shocks per second. After which the tension gradually approached the previous resting value.

Immediately following stimulation to fatigue (at 100 min.) the rate of K^+ efflux increased from a mean resting value of approximately 24 Neq/gm/min to 60 Neq/gm/min. In the remaining sixty minutes, this efflux approached the previous resting value with muscle B (indicated by the squares) recovering more rapidly than muscle A. The ^{14}C Inulin ratio shows negligible gain or loss of water in the system.

This general pattern was observed throughout the series. Table VII illustrates the mean K^+ loss during the three periods of rest, activity and recovery. The K^+ efflux value for recovery represents the K^+ efflux collected during the first collection period following the constant stimulation period.

Table VII illustrates the mean tension values during the periods of rest, activity and recovery. The tension value for recovery represents the tension observed 40 minutes after stimulation to fatigue.

3. Effects of changing bicarbonate concentration

A series of six experiments were performed to test the effect of increasing the external bicarbonate concentration on the rate of K^+ efflux. Fig. 10 shows the changes in tension and K^+ efflux for the mean of the six experiments. The standard error of the mean K^+ efflux is also indic-

Fig. 10

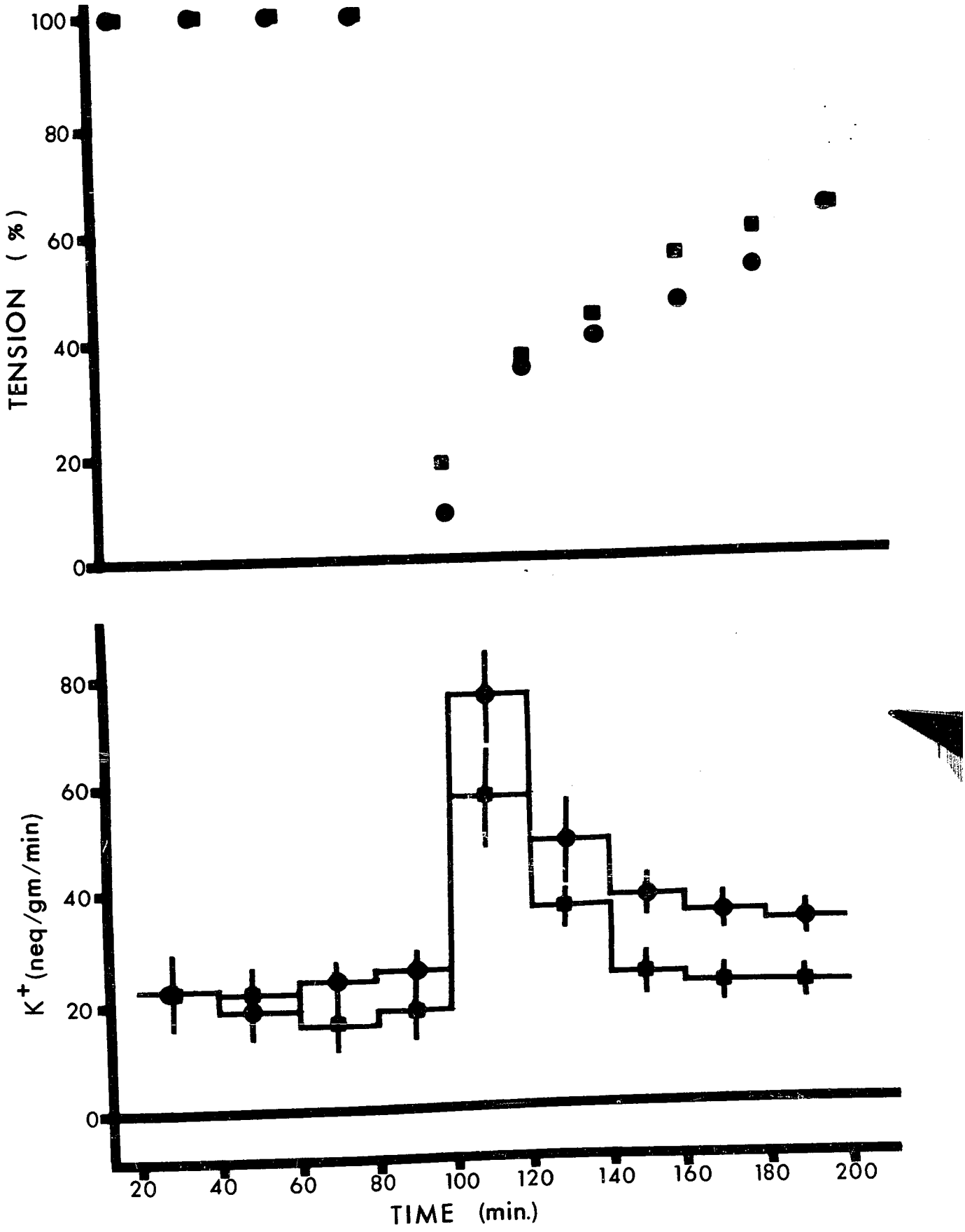
Effects of high external bicarbonate concentration on K^+ efflux and on twitch tension following stimulation to fatigue.

The MEAN and the STANDARD ERROR of six experiments.

Where: Muscle A (■) was superfused with 25 meq/l bicarbonate Ringer's (Solution E, Table IV).

Muscle B (●) was superfused with normal Ringer's (Solution A, Table IV).

Both muscles were gassed with 1% CO_2.


100
80
60
40
20
0
TENSION (%)
80
60
40
20
0
K+ (neq/gm/min)
20
40
60
80
100
120
140
160
180
200
TIME (min.)

ated. The muscles represented by the squares were superfused with 25 meq/1 bicarbonate Ringer's (Solution E, Table IV); those represented by the dots were superfused with normal Ringer's (SOlution A, Table IV) having a bicarbonate concentration of 3.6 meq/1. Both muscles were gassed with 99% O_2, 1% CO_2 giving the high bicarbonate Ringer a pH of 7.98 and the normal Ringer a pH of 7.14. From Fig. 10 it is observed that the muscles buffered with the 25 meq/1 bicarbonate Ringer exhibited a smaller loss in K^+ than those buffered with 3.8 meq/1 bicarbonate. In the case of the muscles superfused with normal Ringer's solution, the net K^+ efflux during recovery is greater than the net K^+ efflux before stimulation by 1,696.80 Neq/gm of muscle. (This figure was arrived at by subtracting the mean value of the resting efflux of three consecutive periods before constant stimulation from each efflux value of four successive periods following constant stimulation including the constant stimulation period and summing the differences.) The same value for the muscles superfused with the high bicarbonate Ringer's is 894.00 Neq/gm. Therefore, these experiments indicate that a high external bicarbonate concentration decreases K^+ efflux upon stimulation to fatigue and during the following recovery period.

It was noticed that in many cases tension recovery was much better in the paired muscle superfused with the high bicarbonate Ringer than the one superfused with the 3.8 meq/1 bicarbonate Ringer. However, quite often the differences in tension recovery were not significant. Further investigation led to the observation that the per cent of tension recovery seemed to vary with the weight of the muscle. Fig. 11

Fig. 11

Graph of per centage recovery versus weight for all bicarbonate experiments.

Where: ● represents muscles superfused with 25 meq/1 bicarbonate Ringer, (Solution E, Table IV).

■ represents muscles superfused with 0.2 meq/1 bicarbonate Ringer, (Solution F, Table IV).

▲ represents muscles superfused with 3.8 meq/1 normal Ringer, (Solution A, Table IV).

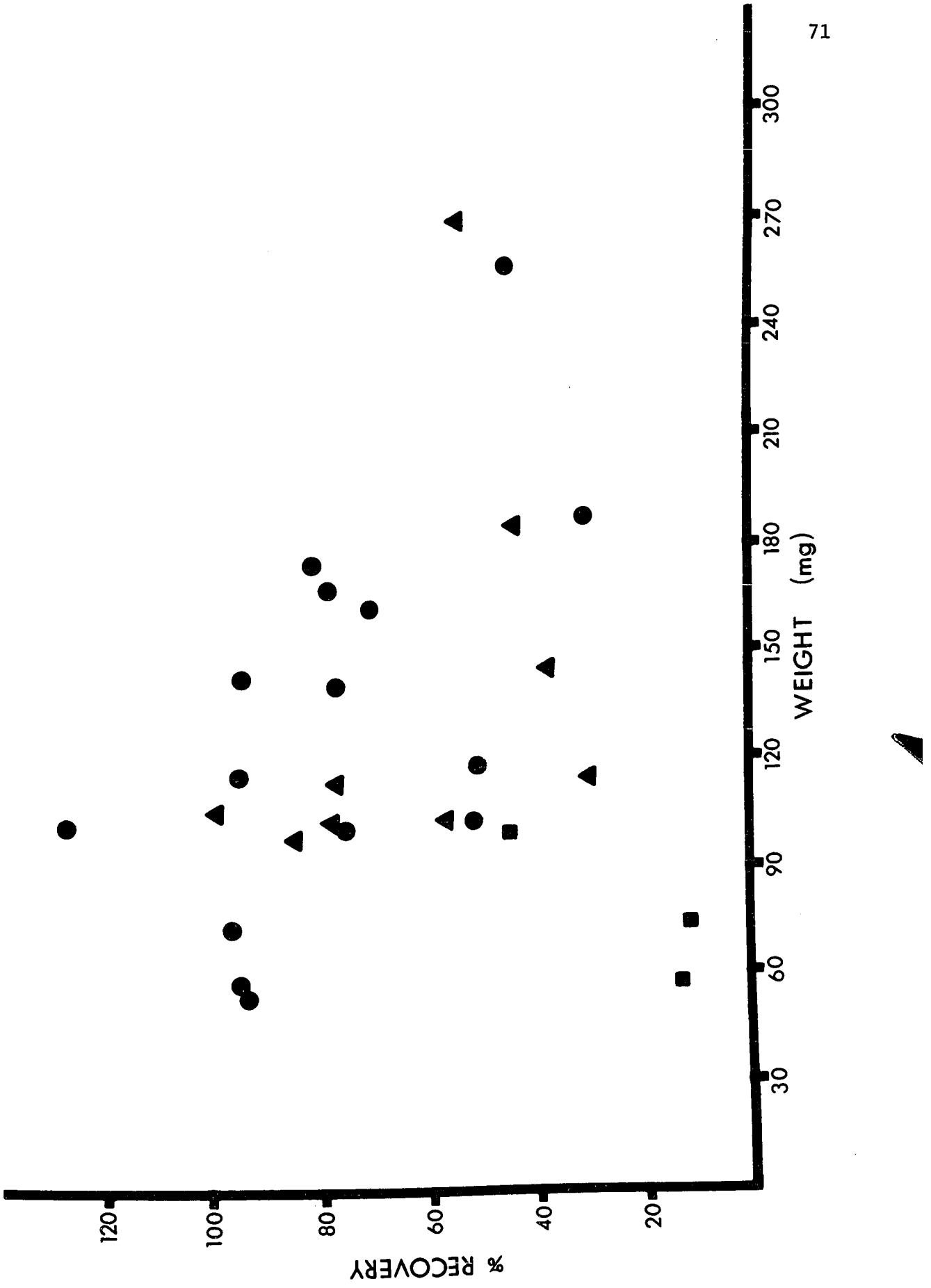

% RECOVERY
120
100
80
60
40
20
WEIGHT (mg)
30
60
90
120
150
180
210
240
270
300

TABLE VIII

Per centage recovery and net K^+ loss according to external bicarbonate concentration and muscle weight.

	Muscle Wt. (mg)	Bicarbonate Conc. (meq)	No. of observ'n	% recovery (80 min)	Net K^+loss (Neq/gm)
A	<100	25.0	3	95.6	84.6
B	<100	3.8	1	84.4	277.6
C	<100	0.2	4	45.5	702.0
D	100=150	25.0	7	81.9	750.7
E	100-150	3.8	6	63.0	1,993.3
F	100-150	0.2	-	-	-
G	>150	25.0	5	51.2	634.5
H	>150	3.8	2	49.1	1,415.2
I	>150	0.2	-	-	-

and Table VIII show that the effect of high bicarbonate on tension recovery and net K^+ loss is greatest in muscles weighing less than 100 mg. The effect of high bicarbonate on tension recovery seems to diminish as the weight of the muscle increases above 150 mg.

In order to observe the effects of a low external bicarbonate concentration on K^+ efflux and tension recovery following stimulation to fatigue, two experiments were performed using 25 meq/l bicarbonate Ringer's (Solution E, Table IV) gassed with 99% O_2, 1% CO_2 (pH 7.98) and 0.2 meq/l bicarbonate Ringer (Solution F, Table IV) gassed with 99% O_2, 1% CO_2 (pH 5.94). The results of both of these experiments indicated a greater K^+ efflux and poor tension recovery in the muscles superfused with the 0.2 meq/l bicarbonate Ringer.

In order to test whether this increase in K^+ efflux observed with the 0.2 meq/l bicarbonate Ringer was due to the low bicarbonate concentration of the Ringer or to the lower external pH produced when this Ringer is gassed with 1% CO_2 an additional experiment was performed. By gassing the 0.2 meq/l bicarbonate Ringer with compressed air it was possible to increase the external pH to 8.9. Thus, in this experiment one muscle was superfused with 25 meq/l bicarbonate Ringer gassed with 99% O_2, 1% CO_2 (pH 7.98) and the other superfused with 0.2 meq/l bicarbonate gassed with compressed air (pH 8.9). The results of this experiment showed a higher K^+ efflux and a slower tension recovery in the muscle superfused with the 0.2 meq/l bicarbonate Ringer.

Fig. 12 shows the results of an experiment using 25 meq/l bicarbonate gassed with 99% O_2, 1% CO_2 (pH 7.98) and 0.2 meq/l bicarbon-

Fig. 12

The results of one experiment contrasting the effects of high and low external bicarbonate concentration on K^+ efflux and twitch tension.

Where: Muscle A (■) was superfused with 25 meq/l bicarbonate solution (Solution E, Table IV).

Muscle B (●) was superfused with 0.2 meq/l bicarbonate Ringer, (Solution F, Table IV).

Muscle A was gassed with 1% CO_2 (pH 7.9); Muscle B was gassed with a mixture of 100% O_2 plus (99% O_2 + 1% CO_2) - pH 7.9.

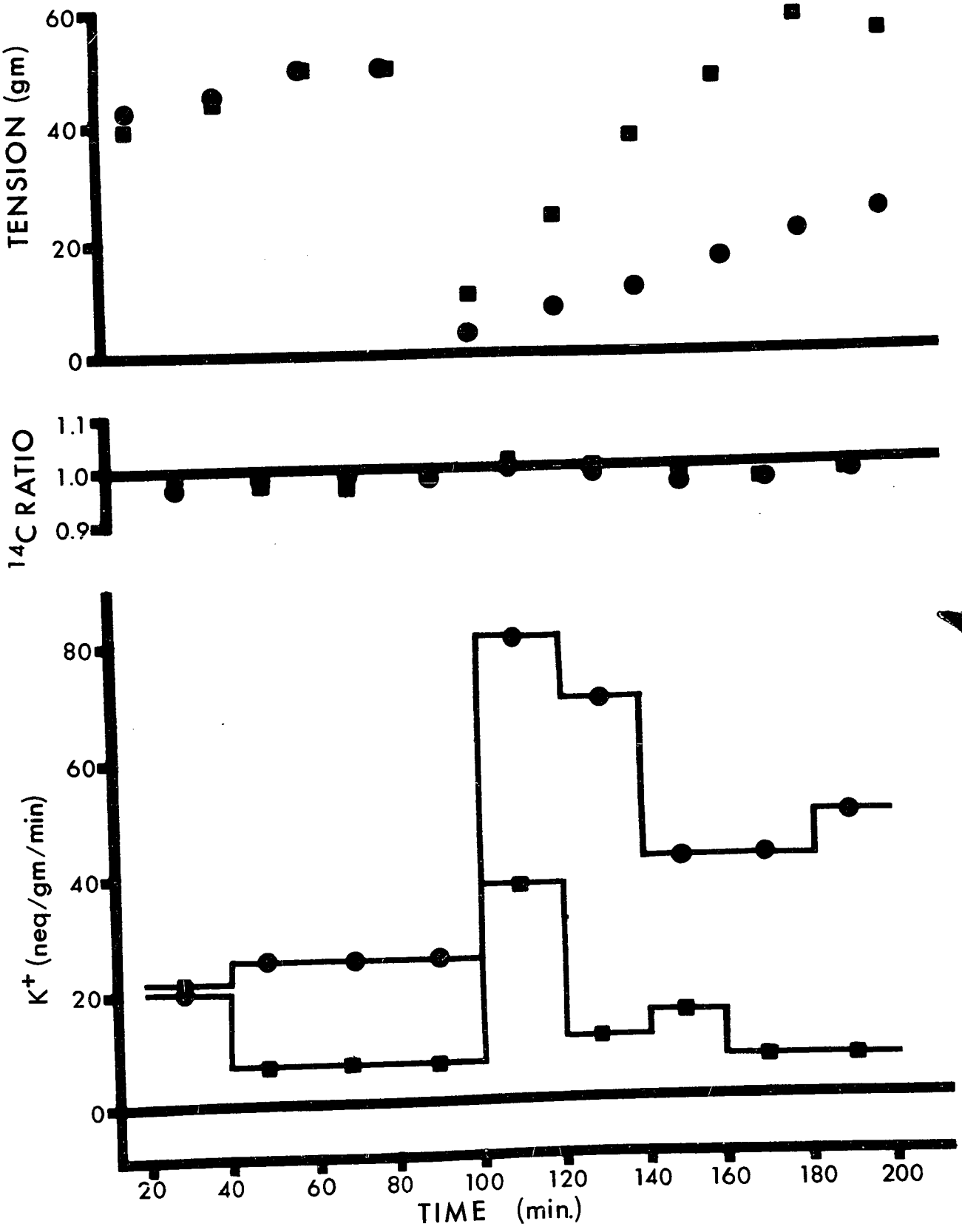

TENSION (gm)
60
40
20
0
^{14}C RATIO
1.1
1.0
0.9
K^+ (neq/gm/min)
80
60
40
20
0
20
40
60
80
100
120
140
160
180
200
TIME (min.)

Fig. 13

Composite histogram of all bicarbonate experiments gassed with 1% CO_2.

Where:  represents 25 meq/l bicarbonate Ringer (6)*

 represents 3.6 meq/l bicarbonate (normal) Ringer (6)*

 represents 0.2 meq/l bicarbonate Ringer (2)*

* denotes number of experiments.

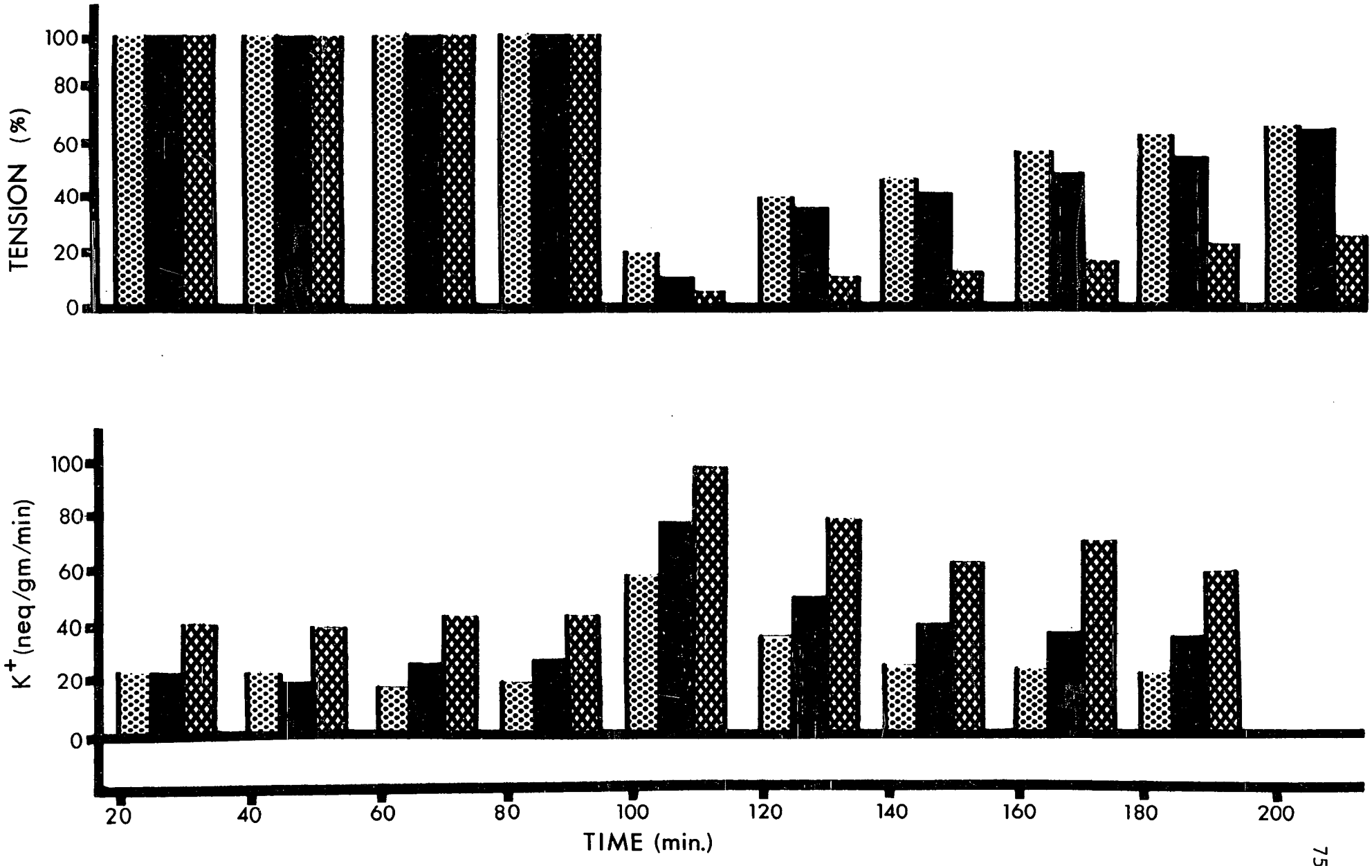

TENSION (%)
100
80
60
40
20
0
K+ (neq/gm/min)
100
80
60
40
20
0
20
40
60
80
100
120
140
160
180
200
TIME (min.)

Fig. 14

Composite histogram of all bicarbonate experiments with the pH of the 0.2 meq/l bicarbonate compensated with low CO_2.

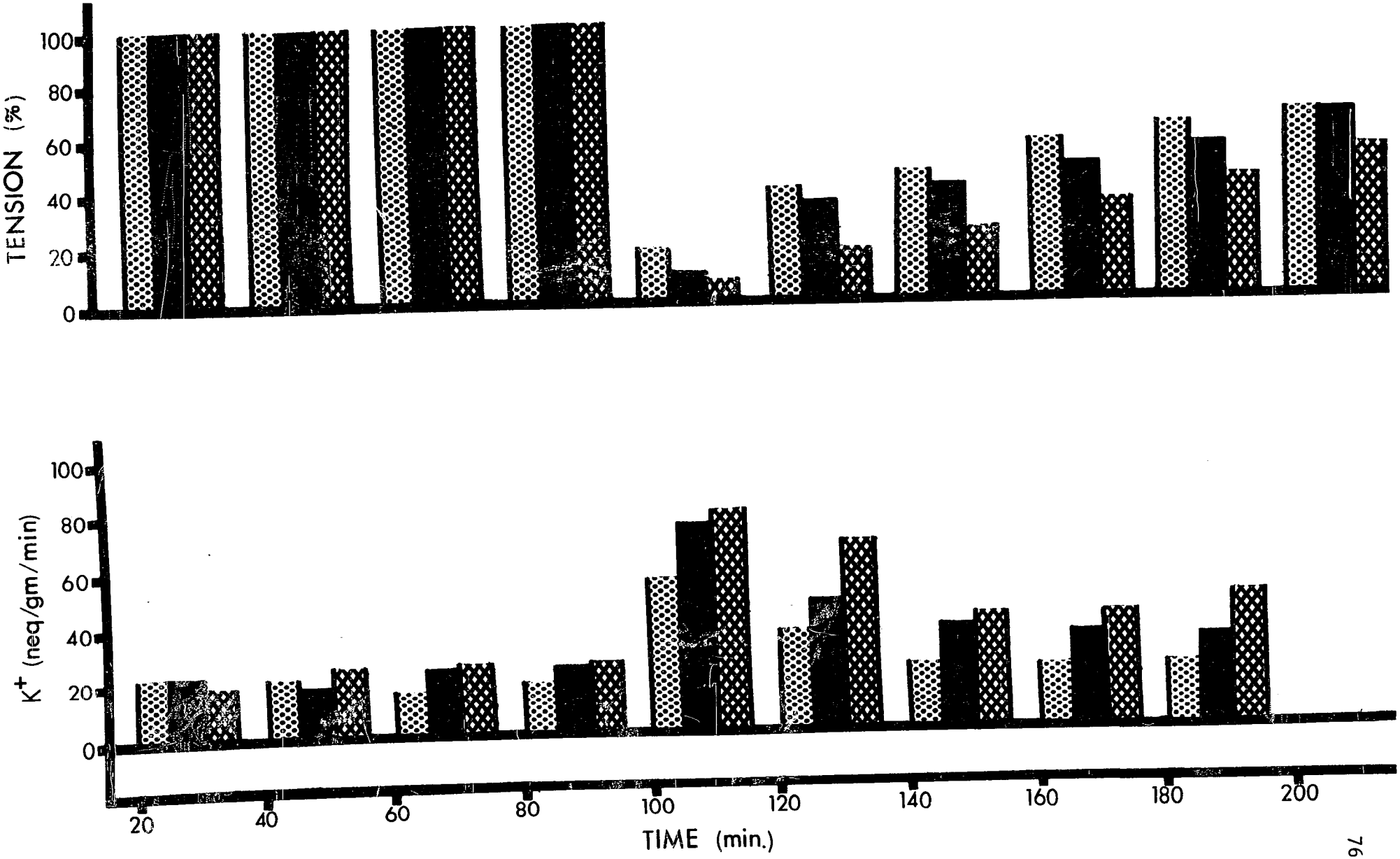
Where:

 represents 25 meq/l bicarbonate Ringer, pH 7.9. (6)*

 represents 3.6 meq/l bicarbonate (normal) Ringer, pH 7.14. (6)*

 represents 0.2 meq/l bicarbonate Ringer, pH 7.9. (1)*

* denotes number of experiments.


TENSION (%)
100
80
60
40
20
0
K+ (neq/gm/min)
100
80
60
40
20
0
20
40
60
80
100
120
140
160
180
200
TIME (min.)

ate gassed with a mixture of 100% O_2 and 99% O_2, 1% CO_2 (pH 7.90). In the case of the muscle superfused with low bicarbonate Ringer the net K^+ efflux during recovery is greater than the net efflux before stimulation to fatigue by 2,793.2 Neq/gm of muscle. While the same value for the muscle superfused with the high bicarbonate Ringer is 552.0 Neq/gm of muscle.

The results of all of these experiments may be summarized in Fig. 13 and 14. Fig. 13 represents the K^+ efflux and per cent tension recovery for all experiments carried out at constant CO_2 (i.e. 1% CO_2). Fig. 14 represents the K^+ efflux and per cent tension recovery for all experiments carried out with the pH of the 0.2 meq/l bicarbonate compensated with low CO_2. Both figures suggest an inverse relationship between net K^+ efflux and tension recovery. An inverse relationship may exist between external bicarbonate concentration and K^+ efflux. There may also be a direct relationship between tension recovery and external bicarbonate. However, when the external pH is maintained within a narrow range (Fig. 14), the effect of low bicarbonate (0.2 meq/l) on K^+ efflux is diminished.

4. Effects of Ouabain, CO_2 and low chloride

a) Effect of Ouabain

Two experiments performed using 3.6 meq/l bicarbonate and 10^{-5} m Ouabain (Solution I, Table IV) showed an increase in K^+ efflux in the Oubain treated muscles. In order to see if this increase in K^+ efflux also occurred in muscles superfused with high bicarbonate Ringer, two experiments were carried out using 25 meq/l bicarbonate Ringer contain-

TABLE IX

The effect of Ouabain on muscles superfused with high bicarbonate Ringer

Ouabain	No. of pairs of muscles	Control K^+ efflux - (neq/gm/min)	Experimental before treatm't K^+ efflux - (neq/gm/min)	Experimental after treatm't K^+ efflux - (neq/gm/min)
10^{-4} m/l	1	18.6 (3)*	17.2 (3)*	76.4 (3)*
$2x10^{-5}$ m/l	2	41.1 (2)*	57.6 (3)*	107.4 (3)*
		27.1 (2)*	22.0 (3)*	66.1 (3)*
	Mean . . .	28.9	32.9	83.3

( )* denotes number of observations.

ing 2 x 10^{-5} m Ouabain and one experiment with the same Ringer and 10^{-4} m Ouabain (Solutions G and H, Table IV). Table IX shows the results of these three experiments. All values of K^+ efflux are for periods prior to stimulation to fatigue. These results show that Ouabain does affect the resting K^+ efflux in muscles washed with 25 meq/l bicarbonate Ringer.

b) Effects of increasing pCO_2

In order to study the effects of increasing pCO_2 on twitch tension and K^+ efflux a number of experiments were carried out using normal Ringer (Solution A, Table IV), gassed with 1, 20, 50 and 100% CO_2. The results of these experiments are shown in Fig. 15. The values given for 1% CO_2 are the mean of six experiments; those for 20% and 50% CO_2 are for one experiment each and those for 100% CO_2 are the mean of two experiments. As the per cent of CO_2 increases, there is a decrease in twitch tension during the three periods of rest, stimulation to fatigue and recovery. The values for K^+ efflux during stimulation to fatigue and recovery seem unduly high in the case of the 20% CO_2. However, in spite of this, there seems to be a general trend of increased K^+ efflux with increasing CO_2 during all three experimental phases.

One experiment was performed in which both muscles were superfused with normal Ringer (Solution A, Table IV) but one muscle was gassed with 100% CO_2 while the other was gassed with 99% N_2, 1% CO_2. In the case of the muscle gassed with 100% CO_2 the net K^+ efflux during recovery was greater than the net K^+ efflux before stimulation to fatigue by 2,345.80 Neq/gm of muscle while the same value for the muscle gassed

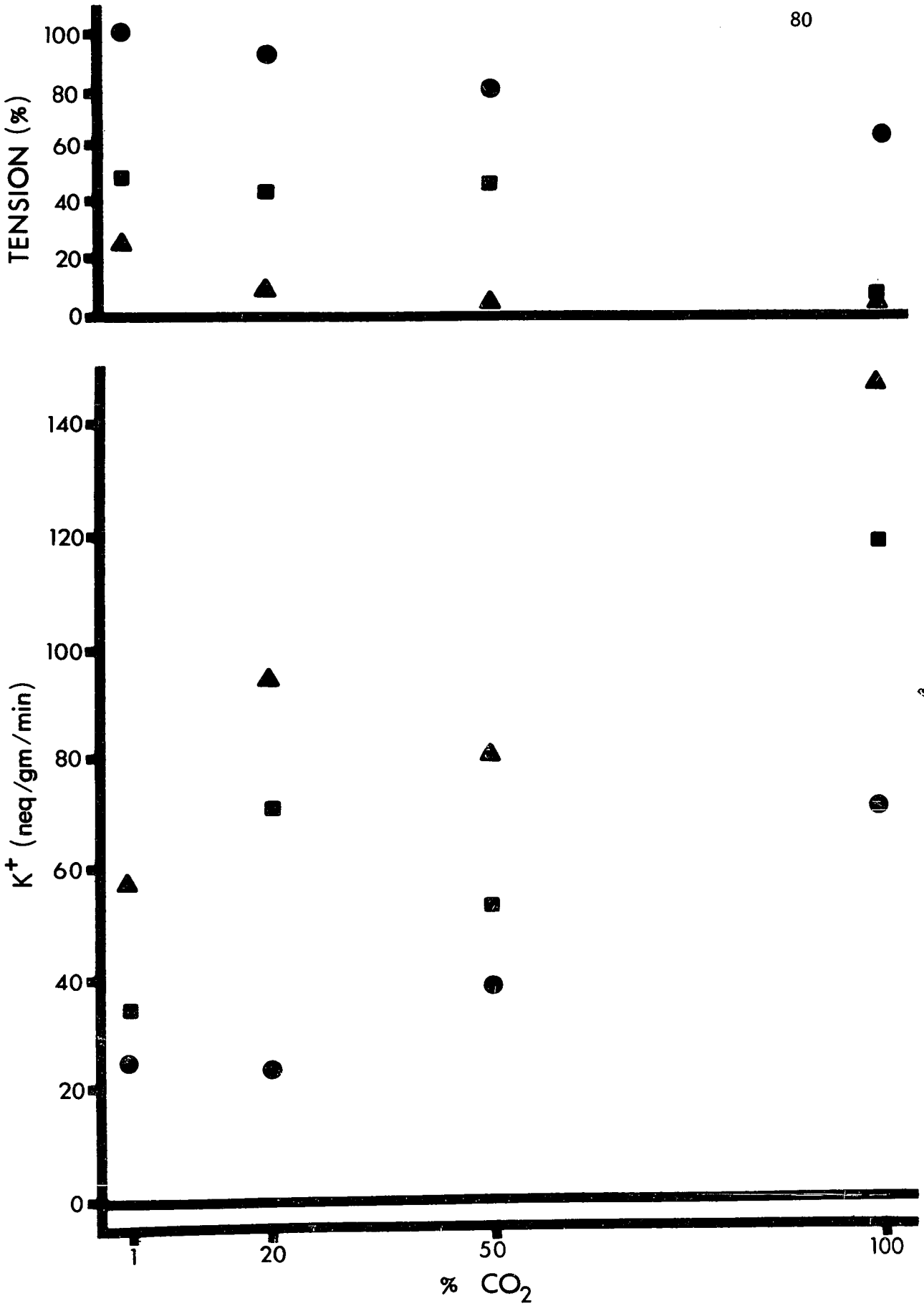
Fig. 15

Effects of varying per centage of CO_2 on K^+ efflux and twitch tension.

Where: ● represent the resting period

▲ represent stimulation to fatigue

■ represent the recovery period
(40 min. after stimulation to fatigue).


TENSION (%)
100
80
60
40
20
0
K^+ (neq/gm/min)
140
120
100
80
60
40
20
0
1
20
50
100
% CO_2

with 99% N_2, 1% CO_2 was 1,616.00 Neq/gm of muscle. This would seem to indicate that the increased K^+ efflux observed at high CO_2 is due to the action of the CO_2 on the tissue and not merely the effect of anoxia.

c) Effect of low chloride

Fig. 16 illustrates the results of one of two experiments performed to study the effects of high carbon dioxide and low chloride on twitch tension and K^+ efflux. There was no stimulation to fatigue in either of these muscles. Both muscles were originally superfused with normal Ringer (Solution A, Table IV), at the end of 60 minutes, muscle A (represented by the dots) was superfused with NaCl deficient Ringer (Solution B, Table IV). Both solutions were gassed with 1% CO_2, 99% O_2 except for the interval 200 - 260 minutes during which time both muscles were gassed with 100% CO_2.

Upon increasing the CO_2 concentration to 100%, the twitch tension of muscle B steadily declined from 45 gm. to approximately 10 gm. after 40 minutes and remained at this level until the CO_2 concentration was returned to 1%. The ^{14}C ratio indicates negligible gain or loss of water by the muscle during this period. During the period of high CO_2 the K^+ efflux of muscle B increased from a mean resting rate of 20 neq/gm/min to almost 60 neq/gm/min. Upon return to 1% CO_2 there was a gradual and almost complete return to the previous resting value.

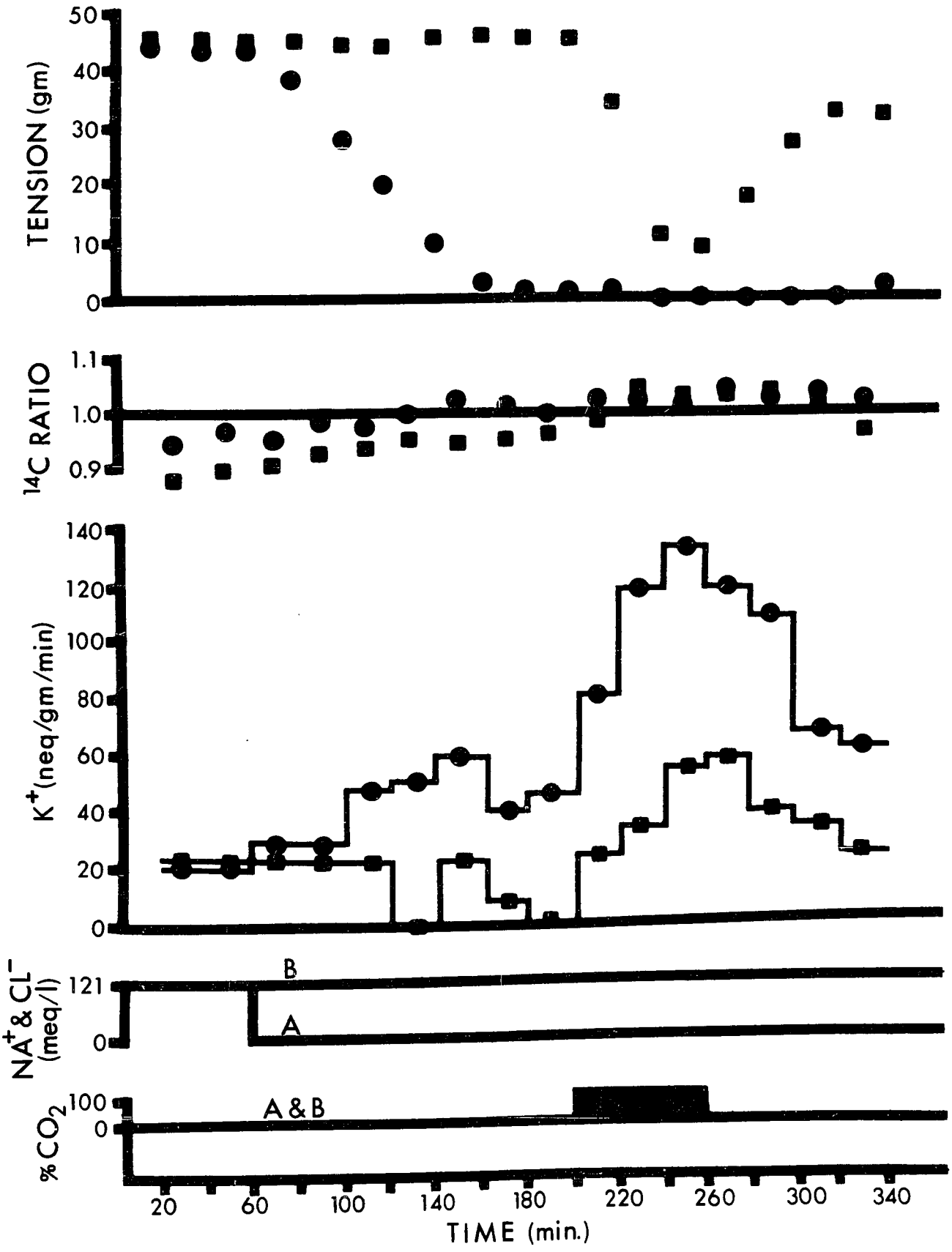
Muscle A was superfused with low chloride Ringer at the end of 60 minutes. After this time the twitch tension gradually decreased almost to zero and remained at this low level for the duration of the

Fig. 16

The results of one experiment showing effects of low chloride and high CO_2 concentrations on potassium efflux and on twitch tension.

Where: ● represents muscle A.

■ represents muscle B.


TENSION (gm)
50
40
30
20
10
0
^{14}C RATIO
1.1
1.0
0.9
K^+(neq/gm/min)
140
120
100
80
60
40
20
0
NA^+ & CL^- (meq/l)
121
0
B
A
% CO_2
100
0
A & B
20
60
100
140
180
220
260
300
340
TIME (min.)

experiment. There was also a steady increase in the rate of K^+ efflux following the change to low chloride Ringer. On increasing the CO_2 to 100%, this rate of efflux was further augmented to almost 140 neq/gm/min followed by a gradual return to its previous resting value of approximately 60 neq/gm/min upon decreasing the CO_2 level to 1%. The ^{14}C ratio suggests that there may have been a slight gain in water by the muscle during the period of high CO_2.

The results suggest that not only is there an increase in the rate of K^+ efflux from the muscle in an atmosphere of high CO_2 but also that this efflux is almost doubled if the muscle is simultaneously superfused with low chloride Ringer.

CHAPTER IV

Discussion

1. Validity of the technique. Net K^+ efflux during rest and stimulation

The validity of this method of measuring continuous ion flux stands or falls on its ability to yield flux measurements of the same order of magnitude as those observed by other investigators employing different techniques. In the first series of experiments in which muscles were superfused with normal Ringer's solution (Solution A, Table IV) and gassed with 99% O_2, 1% CO_2 (pH 7.14), the mean resting K^+ efflux in 12 observations was 0.78 pmole/cm^2/sec (24 neq/gm/min). This value compares favourably with the results of other investigators using ^{42}K tracer and direct K^+ measurement. (See Table III.) The close correlation between the resting K^+ efflux observed with this technique and that of other investigators using whole muscle preparations with the results of Hodgkin and Hororwicz (1959) using a single fiber preparation strongly suggest that muscle can be regarded as being composed of single cellular units each obeying first order permeability kinetics. Furthermore, K^+ movement appears to be membrane limited with K^+ diffusion sufficiently rapid so as not to be rate limiting as indicated by the results of the diffusion study.

During each experiment the muscles were stimulated to fatigue by 2 shocks per second for 200 seconds. This significatnly increased the rate of net K^+ efflux above the resting value of 24.83 neq/gm/min to

57.79 neq/gm/min. This increase of 32.96 neq/gm/min is equal to a net K^+ efflux of 8.6 pmole/cm^2/impulse (measured in muscles superfused with normal Ringer) assuming that a flux of 1 mole/gm/hr is equivalent to a flux of 0.65 pmole/cm^2/sec (Sjodin, 1965). This value compares favourably with the findings of Hodgkin and Horowicz (1959) in single fibers and those of Creese, Hashish and Scholes (1958) in the isolated rat diaphragm preparation. (See Table III.) At the end of this period of constant stimulation the tension of the muscle had decreased to approximately ¼ of its initial tension.

Since these muscles were stimulated to fatigue, one might expect a greater K^+ efflux per impulse than that reported by Hodgkin and Horowicz and Creese et al., due to the development of intracellular acidosis accompanying fatigue. However, the net flux measured here represents the difference between the potassium lost in activity and that pumped back into the cells during recovery whereas, the measurements of Hodgkin and Horowicz and Creese, et al. were based on efflux during activity only.

During recovery the rate of K^+ efflux gradually declined to about 10 neq/gm/min above the resting efflux. Approximately 57 minutes after stimulation to fatigue the twitch tension had recovered to almost 50% of its original value (Table VII). Thus, as the rate of K^+ efflux decreased there was a corresponding increase in twitch tension during the recovery period which usually lasted 80 minutes.

2. Nature of the action of CO_2 and stimulation to fatigue on K^+ efflux

The ability of increasing CO_2 tension to decrease intracellular pH and increase the rate of net K^+ efflux has been documented by a number of investigators studying various tissues including skeletal muscle (Fenn and Cobb, 1934; Hill, 1955; Caldwell, 1958; Kostyuk and Sorokina, 1960). The results of 10 experiments shown in Fig. 15 indicate that resting K^+ efflux is not affected by CO_2 concentrations of 20% or less although a slight decrease in twitch tension may be noticeable. As the per cent of CO_2 is increased however, there is a corresponding increase in net K^+ efflux from the muscles during the periods of rest, fatigue and recovery. This increased K^+ loss by the muscle is accompanied by a corresponding decrease in twitch tension such that at 100% CO_2 there is only slight tension recovery following stimulation to fatigue.

One experiment performed showed that the increase in K^+ efflux observed with 100% CO_2 was greater than that observed in the other muscle of the pair gassed with 99% N_2, 1% CO_2. This would seem to indicate that the increased K^+ efflux observed with increasing CO_2 tension is due to the decrease in intracellular pH brought about by the CO_2 and not merely due to the effects of anoxia on the muscle. This is in keeping with the findings of Hill (1955) who showed that muscles stimulated (but not fatigued) in a medium saturated with 100% CO_2 and buffered with bicarbonate seldom showed any evidence of lactic acid formation.

The increased K^+ efflux observed in isolated muscles immersed in saline and subjected to high CO_2 tension may possibly be explained by the difference in the buffer capacity of the muscle tissue and the saline

medium (Fenn, 1928, 1956). Assuming the intrinsic buffer capacity of the extracellular fluid is much smaller than that of muscle tissue, increased CO_2 tension should cause a greater decrease in extracellular fluid pH than intracellular pH. In the muscle's attempt to restore equilibrium there would be an exchange of intracellular cation for excess extracellular H^+ ion. K^+ being the most abundant intracellular cation would be the logical ion to exchange for H^+. Therefore, K^+ would tend to move toward that phase where there is a greater fall in pH, i.e. into the extracellular fluid. During recovery K^+ may re=enter the fibers in exchange for H^+.

The increased rate of K^+ efflux and decreased tension recovery associated with stimulation to fatigue may also be explained on this basis. During constant stimulation H^+ ions may build up inside the muscle fibers displacing K^+ and temporarily exceeding the intracellular buffering capacity. This decrease in pH may also extend to the extracellular fluid. During the recovery process K^+ might re-enter the fibers in exchange for H^+ possibly by means of a pump as described in Fig. 2 b.

3. <u>The effect of varying the external bicarbonate concentration on K^+ efflux and tension recovery</u>

If the explanation above is correct it seems reasonable that an increase in the buffer capacity of the extracellular fluid should bring about a decrease in the rate of K^+ efflux and better tension recovery following stimulation to fatigue. Fig. 10 illustrates the results of six experiments in which muscles superfused with 25 meq/l

bicarbonate Ringer gassed with 1% CO_2 (pH 7.14) did show a significant decrease in the rate of K^+ efflux compared with the other member of each pair superfused with 3.6 meq/l bicarbonate Ringer and gassed with 1% CO_2 (pH 7.98). The results of four experiments performed with 25 meq/l bicarbonate Ringer and 0.2 meq/l bicarbonate Ringer showed a definite increase in K^+ efflux and poorer tension recovery in the muscles superfused with the low bicarbonate Ringer although the external pH of the solution was identical to that of the high bicarbonate Ringer. (See Fig. 12.)

A comparison of Fig. 13 and Fig. 14 shows that as per cent of CO_2 gassing the 0.2 meq/l bicarbonate Ringer was decreased so that its pH could be increased to the level of the 25 and 3.6 meq/l bicarbonate Ringer its buffering capacity was increased. Because of this the difference in K^+ efflux and tension recovery between the 3.6 meq/l bicarbonate Ringer and the 0.2 meq/l bicarbonate Ringer is not as great in Fig. 14 as it is in Fig. 13. Fig. 11 and Table VIII show that the effect of high bicarbonate on tension recovery and net K^+ loss is greatest in muscles weighing less than 100 mg. The effect of high bicarbonate on tension recovery seems to diminish as the weight of the muscle increases. This would account for the fact that the increase in tension recovery of the muscles superfused with 24 meq/l bicarbonate Ringer is not significantly different from the tension recovery of the muscles superfused with 3.6 meq/l bicarbonate Ringer in Fig. 10 since this figure is based on the mean of six experiments in which the weight of the muscles varied between 90 and 160 mg.

Thus, it would appear that for a given amount of bicarbonate delivered to the muscle from the superfusion solution a smaller muscle is able to use this bicarbonate more readily. This difference may be due to the increased volume of extracellular fluid in the heavier muscles and possibly a difference in diffusion rates.

From the results of these experiments it would appear that the rate of K^+ efflux and tension recovery following stimulation to fatigue are affected by the bicarbonate concentration of the superfusing solution. A possible explanation for this effect of bicarbonate on the $K^+ - H^+$ exchange may be as follows. It seems that the most important factor in recovery from intracellular acidosis is keeping the electrochemical gradient for H^+ across the membrane as low as possible. Therefore, when the external bicarbonate concentration is low as soon as H^+ ions leave the fibers they build up in the extracellular fluid and this build-up tends to slow down the net outflow. If however, a high bicarbonate concentration exists in the extracellular fluid the H^+ ions could be converted to CO_2 as soon as they cross the membrane. Hence no opposing gradient can develop and a pump such as that proposed in Fig. 2 b could continue to push H^+ ions out of the fibers in exchange for K^+ unimpeded.

4. A possible explanation for the action of low chloride Ringer on K^+ efflux

These experiments were carried out to test a hypothesis previously proposed to explain the effects of CO_2 on transmembrane potentials (Mainwood, 1966). It was shown that transmembrane potentials in muscle fibers

decrease as the per centage of CO_2 in the gas phase increases beyond 20%. This effect is greatly exaggerated in a chloride-free medium so that the cell membrane acts almost as a CO_2 electrode. It was proposed that the depolarization is due to a current flow generated by the efflux of HCO_3^- ions and that anion competition in the membrane normally reduces this to very low levels but that in the absence of chloride, HCO_3^- permeability increases and so the depolarizing current in response to CO_2 is enhanced. A corollary of this is that above 20% CO_2, there should be an increase in net K^+ efflux and that in the absence of chloride the K^+ efflux should be considerably greater. The two experiments performed showed that the rate of K^+ efflux in muscles exposed to 100% CO_2 is almost doubled if the muscle is superfused with low chloride Ringer (as in Fig. 16).

5. The action of Ouabain on the resting K^+ efflux of muscles superfused with high bicarbonate Ringer

Ouabain (10^{-5}M) applied to muscles superfused with normal Ringer showed a significant increase in resting K^+ efflux. Table IX shows that 10^{-4} M Ouabain applied to muscles superfused with 25 neq/l bicarbonate Ringer (Solution E, Table IV) caused almost a five fold increase in resting K^+ efflux. Upon decreasing the concentration to 2 x 10^{-5} m the increase in K^+ efflux in the Ouabain treated muscle was still twice that of its untreated mate. These increases are in keeping with obser- vation of Harris (1957). As mentioned earlier Sjodin and Beaugé (1968) found that K^+ efflux was dependent upon intracellular Na^+ concentration with no change in K^+ efflux in muscles treated with 10^{-5} M strophan-

thidin having an intracellular Na^+ concentration of 5 mMoles/Kg fibers. The intracellular Na^+ concentration of the muscles used in the experiments reported in Table IX was undoubtedly well above this minimum. Thus, increasing the external bicarbonate concentration does not seem to alter the effect of Ouabain on resting K^+ efflux. Since Ouabain is capable of blocking the Na^+ - K^+ exchange mechanism and if H^+ ions travel via the same carriers as proposed in Fig. 2 b, it should be possible to alter the buffering capacity of muscle through the action of Ouabain.

6. A model for muscular fatigue

It was mentioned earlier that since both acidosis and anoxia are likely to accompany fatigue it may be possible to simulate fatigue by means of intracellular acidosis and anoxia. From the preceeding results it is evident that concentrations of CO_2 greater than 20% are capable of increasing K^+ efflux and decreasing twitch tension even in resting muscle. When an isolated resting muscle is subjected to increased pCO_2, the H^+ ions formed are probably first buffered in the extracellular fluid however, once this buffer supply is exhausted, it remains for the intracellular proteins to buffer the excess acid. During this process H^+ may come to occupy the fixed anionic charge sites previously held by K^+. Thus K^+ ions move out of the cell into the extracellular fluid with the simultaneous exit of an anion to retain electroneutrality. That HCO_3^- may be the anion in question seems possible from the hypothesis of Mainwood (1966) and the observations presented herein.

Likewise when a muscle is stimulated to fatigue H^+ ions are produced not only by the excess CO_2 present but also from the dissociation of the various metabolic end-products such as lactic acid. If the buffering capacity of the extracellular fluid is extended by increasing its bicarbonate content less of the buffering will have to be done by the intracellular buffer system and hence less K^+ will be displaced by H^+.

In both instances the recovery process will be the same, i.e. the expulsion of H^+ from the cell with the readmittance of K^+. This could be brought about by some form of $H^+ - K^+$ exchange process. As previously described, Ouabain is capable of blocking the $Na^+ - K^+$ pump in the presence of normal and high bicarbonate Ringer. If H^+ and Na^+ are extruded from cells by a common transport mechanism such as that proposed in Fig. 2 b, it should be possible to alter the cell's ability to recover from intracellular acidosis through the application of Ouabain. Although some preliminary experiments by Bondini and Withrow, 1965 seem to support this hypothesis more extensive investigation may help determin whether H^+ ions are actively transported.

Summary

1. A new technique for measuring net K^+ flux between frog sartorious muscle fibers and the external solution has been developed.

2. Under experimental conditions in a resting muscle the net K^+ efflux is approximately 24 neq/gm/min or 0.78 pmole/cm^2/sec.

3. This K^+ efflux increases upon stimulation to fatigue to approximately 60 Neq/gm/min or 8.6 pmole/cm^2/impulse.

4. When the buffering capacity of the extracellular fluid is extended by increasing the bicarbonate content of the superfusion fluid the net K^+ efflux observed during stimulation to fatigue and recovery is decreased. The extent of this decrease is dependent upon the weight of the muscle with muscles weighing less than 100 mg exhibiting the greatest decrease in K^+ efflux.

5. With increased CO_2 levels (100%) the resting K^+ efflux is increased to 60 Neq/gm/min and in the absence of Na^+ and Cl^- the efflux is further augmented to almost 140 Neq/gm/min without stimulation.

6. Ouabain in concentrations of 2×10^{-5} M and greater is capable of doubling the net K^+ efflux in resting muscles superfused with high bicarbonate Ringer's solution.

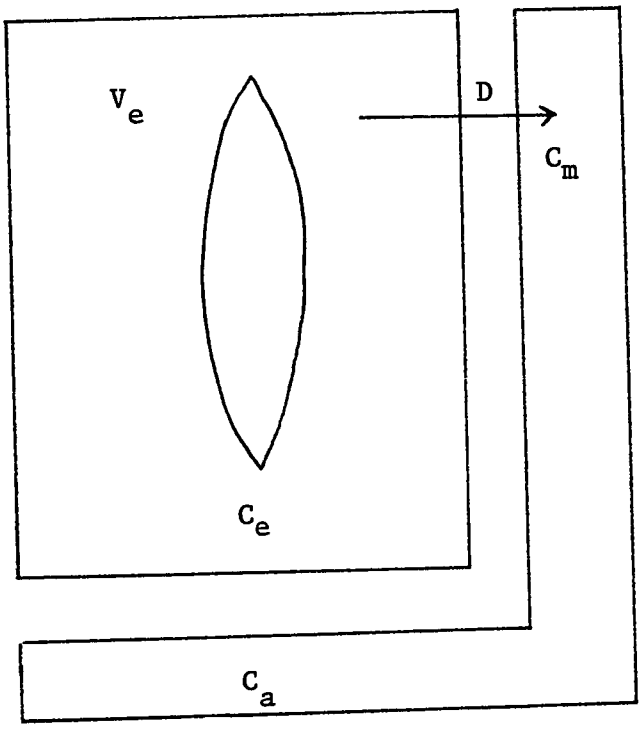
7. A net $H^+ - K^+$ exchange mechanism such as that proposed in Fig. 2 b may be used to explain the recovery process following stimulation to fatigue, i.e., H+ ions transported out of the cell where they are buffered mainly by HCO_3^- in the extracellular fluid while K^+ are

returned to their previously held fixed-anionic sites within the cell.

8. The possibility of using high pCO_2 to simulate muscle fatigue has been discussed on the assumption that intracellular acidosis plays a primary role in the development of fatigue.

APPENDIX

The results of the diffusion study were interpreted on the basis of the following model.



Where:

C_e^o = initial concentration in extracellular fluid at time zero.

C_e^t = concentration in extracellular fluid at any instant during washout.

C_o = concentration in superfusion solution (zero in the simplest case).

C_m = mean concentration in surface fluid.

C_a = concentration in collected fluid.

F = superfusion flow rate.

V_e = volume of extracellular fluid.

D = diffusion index, a measure of the "diffusing efficiency" of the system measured in units of flux rate/concentration difference.

In general the rate of transfer of any substance from the extracellular fluid (V_e) will be equal to its concentration difference across the membrane multiplied by its diffusing efficiency (D) i.e.

$$-\frac{dQ}{dt} = (C_e{}^t - C_m) \times D \qquad \ldots\ldots\ldots\ldots \quad (1)$$

In a steady state the rate of transfer of substance across the membrane will equal the rate of measured efflux. i.e.

$$-\frac{dQ}{dt} = F\ (C_a - C_o) \qquad \ldots\ldots\ldots\ldots \quad (2)$$

If now we consider the case for Na^+ washout, at time zero, the initial concentration in the extracellular fluid ($C_e{}^o$) will be equal to the concentration in the superfusion fluid before time zero; i.e. 120 meq/l. From time zero on $C_e{}^o$ becomes $C_e{}^t$ and will decrease at a rate inversely proportional to V_e. i.e.

$$\frac{dC_e{}^t}{dt} = \frac{dQ}{dt} \times \frac{1}{V_e} \qquad \ldots\ldots\ldots\ldots \quad (3)$$

reaching zero when $\frac{dQ}{dt} = 0$.

Thus knowing V_e, $C_e{}^t$ can be calculated from instant to instant. From (1) and (2) the following equation is obtained:

$$(C_e{}^t - C_m) \times D = F\ (C_a - C_o).$$

Suppose C_o is 0 and the flow rate is kept high so that C_m is very small compared to C_e^t; then, in simplest form -

$$C_e^t \times D = F\, C_a$$

and from (3) -

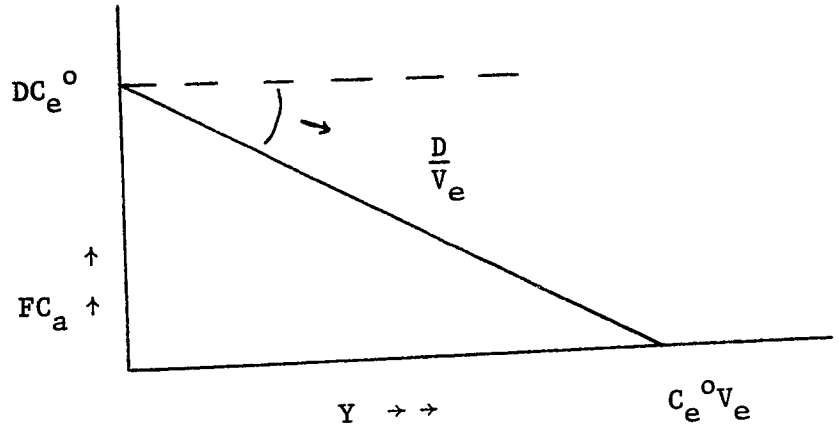
$$C_e^t = C_e^o - {}_o\int^t dQ \,.\, dt \times \frac{1}{V_e}$$

Now ${}_o\int^t dQ \,.\, dt$ is the cumulative amount of substance collected at time t. Let us call this Y.

Thus, the relationship becomes -

$$F\, C_a = D\,(C_e^o - \frac{Y}{V_e})$$

Plotting $F\, C_a$ against Y we get a linear relationship -



When t = o, Y = o and $F\, C_a = D\, C_e^o$

When t = ∞, $F\, C_a$ = o and $C_e^o = \frac{Y}{V_e}$

V_e values can be compared with ^{14}C Inulin space value and C_e^t can be compared with tension values.

The diffusion index (D) may be calculated from either the Y intercept or the slop of Fig. 8.

When $\frac{dC_e^t}{dt}$ falls to a low value and becomes fairly constant it can be assumed that a "semi-steady state" condition has been reached where for a time the rate of Na^+ leakage from cells balances the rate of loss from the extracellular fluid to the perfusion fluid. If the rate of loss of Na^+ can now be altered by changing the rate of superfusion, a third method of estimating D may be employed.

The rate of efflux (R) at a given flow rate (F), will be given by

$$R = D (C_e^o - C_e^{tx})$$

Suppose F_1 is decreased to F_2 so that C_e^{tx} approaches a new value C_e^{ty}, then R will decrease to R^1 such that

$$R^1 = D (C_e^o - C_e^{tx})$$

And since

$$R - R^1 = D (C_e^o - C_e^{tx}) - D (C_e^o - C_e^{ty})$$

$$= D (C_e^{ty} - C_e^{tx})$$

therefore,

$$D = \frac{R - R^1}{C_e^{ty} - C_e^{tx}}$$

At time 180, the superfusion flow rate was decreased from 0.194 ml/min to 0.019 ml/min. At time 180, the rate of efflux (R) was 150 neq/min and C_e^{tx} was 0.77 meq/1.

At time 200, the rate of efflux (R^1) was 77 neq/min and C_e^{ty} was 4.00 meq/1. Substituting these values in the equation above and solving for D, we get a value of 23 neq/min per gradient of one meq/1. From the slop of Fig. 8, we get a value of 4.7 neq/min per gradient of one meq/1. From the Y intercept of Fig. 8, we get a value of 8 neq/min per gradient of one meq/1.

The value obtained using the steady state equation is probably an overestimate due to the increased Na^+ concentration in C_e as Na^+ leaked out of the muscle fibers. The value of 4.7 neq/min per gradient of one meq/1 is probably an underestimate due to the constantly decreasing value of C_e^t and the steady release of Na^+ from the fibers.

The most favourable value is that obtained from the Y intercept dur to the fact that the Na^+ efflux from the fibers would be negligible at this time and C_e^t had not started to decrease.

It was mentioned earlier that Na^+ was chosen as the washout ion because a measure of the mean tissue sodium could be estimated independently by relative twitch tension. This property was applied in the following way: at the end of the washout period Na^+ was put back into the muscle by applying superfusion solutions containing 20,50 and 121 meq/1 Na^+. From the curve at the R.H.S. of the figure (Fig. 17) it was determined that an increase of 1.6 meq/1 of Na^+ caused a tension increase

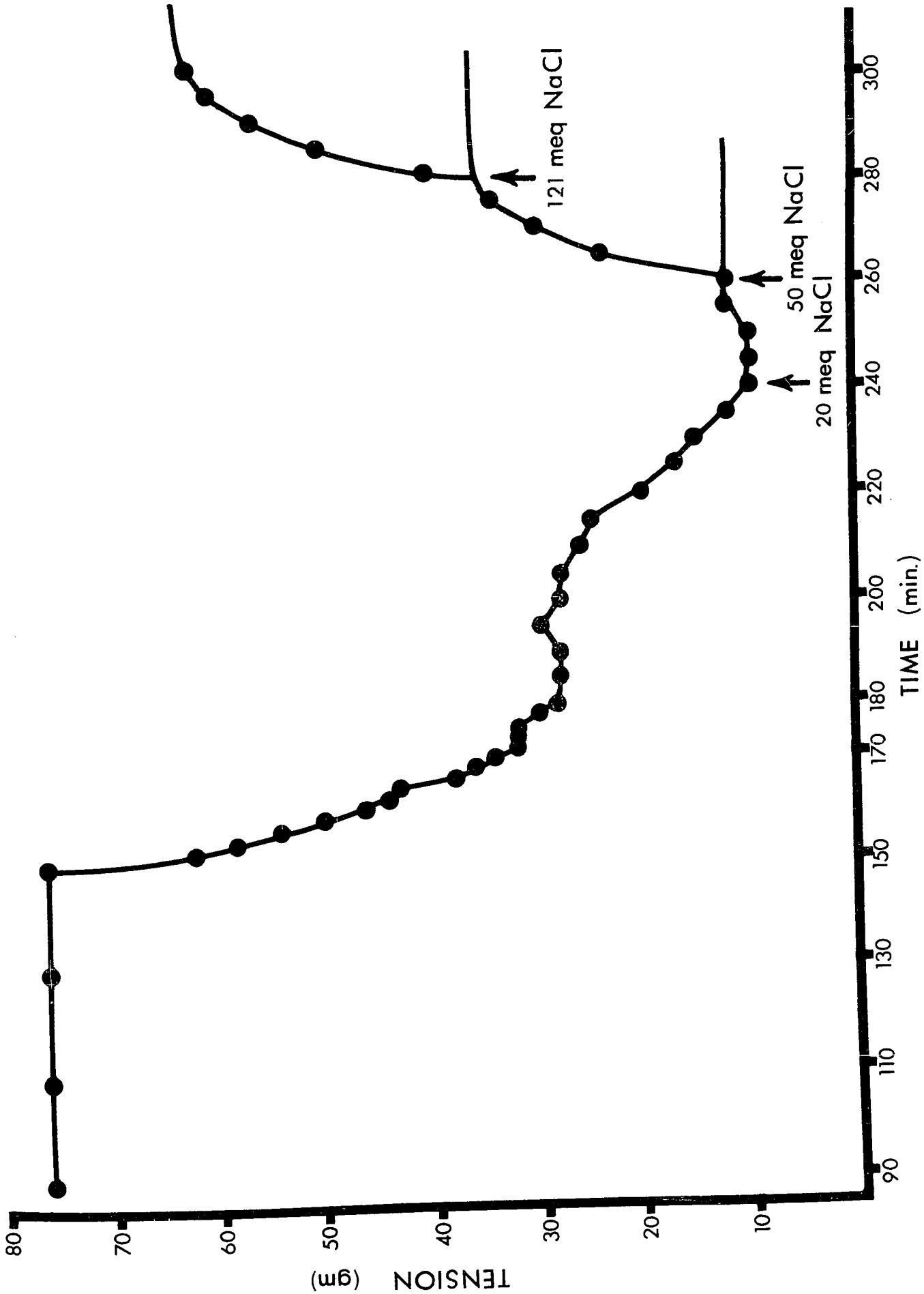
of 1 gm. Thus knowing that 1 gm. of tension represents approximately 1.6 meq/l internal sodium this value could be used to determine the Na^+ content of the muscle at any given tension during the washout of the E.C.F. at any given time represented by the L.H.S. portion of Fig. 17. The values for internal Na^+ content at a given time during the washout determined this way were found to agree quite favourably with the amount determined by subtracting the cumulative total washout at that time from the original Na^+ concentration of the extracellular fluid determined as mentioned previously.

Fig. 17

Tension versus time graph.

Diffusion study.

Sodium washout experiment.


121 meq NaCl
50 meq NaCl
20 meq NaCl
TENSION (gm)
TIME (min.)
10
20
30
40
50
60
70
80
90
110
130
150
170
180
200
220
240
260
280
300

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